VASCULAR BIOLOGY IN THE MENSTRUAL CYCLE, PREGNANCY AND PRE-ECLAMPSIA

BY

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ABSTRACT

BACKGROUND Thrombotic disease is rare but significant in women of reproductive age. Thrombosis and thromboembolism are the most common causes of maternal death in the United Kingdom. Pre-eclampsia is associated with increase in immediate and lifetime risk of adverse thrombotic events. Both pregnancy and the follicular phase are associated with arterial thrombosis and myocardial infarction. Modulation of haemostatic mechanisms in reproductive physiology and disease is not well understood. Outwith pregnancy, stimulated tissue plasminogen activator (t-PA) release, platelet activation, arterial stiffness and circulating endothelial progenitor cell (EPC) number are established indicators of vascular pathology and prognosis.

AIMS (i) To compare endogenous fibrinolysis between pregnant women and non-pregnant control women. (ii) To make serial measurements of reproductive hormones, inflammatory mediators, platelet and monocyte activation, arterial stiffness and circulating EPCs throughout the normal menstrual cycle, healthy pregnancy, and pregnancy affected by pre-eclampsia.

METHODS (i) Endogenous fibrinolytic capacity was assessed using forearm venous sampling and plethysmography during intra-arterial infusion of bradykinin (a known stimulant of endothelial t-PA release). Healthy women in the third trimester were recruited from antenatal clinics. Healthy volunteers were studied in their follicular phase. (ii) Platelet-monocyte aggregates and surface markers of platelet and monocyte activation were assessed with flow cytometry. Reproductive hormones, inflammatory mediators (soluble intercellular adhesion molelcule-1 (ICAM-1), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α)) and soluble markers of platelet activation were measured by enzyme-linked immunosorbent assay (ELISA). Arterial stiffness was derived using pulse wave analysis (PWA) and pulse wave velocity (PWV). Circulating EPCs were assessed by flow cytometry and cell culture. All measurements were taken longitudinally in three different populations: healthy women during a single menstrual cycle (4 time points), healthy pregnant women (4 time points and post-partum) and women with pre-eclampsia (at diagnosis and post-partum).

RESULTS (i) Pregnant women had more plasminogen activator inhibitor type 1 (PAI-1) antigen and lower active t-PA plasma concentrations than non-pregnant women. (ii) Pregnant women had greater platelet and monocyte activation, plasma soluble ICAM-1 and IL-6 than non-pregnant women. There was no difference in platelet activation or inflammatory mediators between healthy and pre-eclamptic pregnant women. Systemic arterial stiffness varies during the menstrual cycle. In pregnancy both systemic and central arterial stiffness are lowest during the second trimester. Arterial stiffness was greater in pre-eclampsia and this persisted post-partum, despite blood pressure returning to normal. Concentration of EPCs (cytometry) varied during the menstrual cycle and was greatest in the follicular phase. Endothelial progenitor cell colony formation was reduced in healthy pregnancy compared to the follicular phase. There was no difference in either EPC assay between healthy and pre-eclamptic pregnant women.

CONCLUSIONS Important constituents of thrombotic and inflammatory activity are observed to vary with normal changes in reproductive status. No difference was observed in these factors between healthy pregnant and pre-eclamptic women. Greater arterial stiffness was observed in pre-eclamptic women, also continuing after pregnancy. This may contribute to the increased immediate and lifetime risk of vascular disease in these women. Variation in circulating EPCs during the menstrual cycle may be due to a role in endometrial angiogenesis.

To Nick and Sam

CONTENTS

ABBREVIATIONS

AcLDL	acetylated low-density lipoprotein
AIx	augmentation index
ANOVA	analysis of variance
APC	allophycocyanin
BD	Becton Dickinson
bpm	beats per minute
BMI	body mass index
CD	cluster of differentiation
CFU-EPCs	colony forming unit-endothelial progenitor cells
CV	coefficient of variation
D	day
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
dias BP	diastolic blood pressure
Dil-AcLDL	1,1'-dioctadecyl-3,3,3',3'-tetramethylcarbocyanine-labelled acetylated LDL
Dil	dilution
ECCM	EndoCult™ complete culture medium
ECM	extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	**E**nzyme-**L**inked **I**mmunosorbent **A**ssay
EPC	endothelial progenitor cell
ER	oestrogen receptor
FBF	forearm blood flow
FITC	fluorescein isothiocyanate
FL	fluorescence parameter
FSC	forward-scatter
FSH	follicle stimulating hormone
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GP	glycoprotein

GTN	glyceryl trinitrate
Hct	haematocrit
HLA	human leucocyte antigen
HSCs	haematopoietic stem cells
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
ISSHP	**I**nternational **S**ociety for the **S**tudy of **H**ypertension in **P**regnancy
IU	international units
JAM	junctional adhesion molecule
KDR	kinase insert domain receptor
L	ligand
LDL	low-density lipoprotein
LH	lutenising hormone
m/s	metres per second
Mac-1	membrane-activated complex-1
MCP	monocyte chemo tactic protein
MFI	mean fluorescent intensity
mL	millilitre
NF-kβ	nuclear factor kappa β
NP	non-pregnant
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor type 1
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PF	platelet factor
PP	post-partum
PSGL-1	P-selectin glycoprotein ligand-1

PWA	pulse wave analysis
PWV	pulse wave velocity
RANTES	**R**egulated upon **A**ctivation, **N**ormal **T**-cell **E**xpressed, and **S**ecreted
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
SDF-1	stromal cell-derived factor-1
SEM	standard error of the mean
SNP	sodium nitroprusside
SSC	side-scatter
sys BP	systolic blood pressure
TM	thrombomodulin
TNF-α	tumour necrosis factor alpha
t-PA	tissue plasminogen activator
UEA-1	Ulex europaeus agglutinin-1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
vWF	von Willebrand factor

DECLARATION

Except where due acknowledgment is made by reference, the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

Chapter 3

I acknowledge Sharon Cameron and her nurses from the Wellcome Trust Clinical Research Facility for their assistance in performing these studies. I acknowledge Mrs. Pamela Dawson, who performed the t-PA and PAI-1 ELISAs.

Chapter 4

I acknowledge the assistance of Dr Jehangir Din, who designed the platelet activation protocols; Imogen Smith, a medical student who performed the staining and analysis of the samples from the non-pregnant group; and Jamshid Pourazar, who performed the ELISAs. Ms Mary Stoddart performed the hormone immunoassays.

Chapter 5

I acknowledge the assistance of Dr Olga Tura and Dr Nicholas Mills, who designed the EPC staining protocols; and Amie Bowler, who helped establish the CFU-EPC assay in our laboratory. Imogen Smith performed the flow cytometry and preliminary culture of samples from the non-pregnant group and Abigail Short; another medical student, performed the immunofluorescence of the CFUs. Ms Rose Leask performed the VEGF ELISA and the preliminary culture of the CFU-EPC samples. I acknowledge Jamshid Pourazar, who performed the inflammatory ELISAs.

Chapter 6

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All chapters (3,4,5 & 6)

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CHAPTER 1

INTRODUCTION

Robb AO, Mills NL, Newby DE, Denison FC.
Endothelial progenitor cells in pregnancy.
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OVERVIEW

The endothelium plays a central role in vascular homeostasis. Female sex hormones affect vascular health, with oestrogens in particular having protective effects on vascular structure and function. During the reproductive years, female sex steroids fluctuate cyclically during the menstrual cycle and increase dramatically in pregnancy. Thrombotic disease is rare but significant in women of reproductive age. For example: myocardial infarction is more common in the follicular phase of the menstrual cycle, pregnancy is associated with an increased risk of arterial and venous thrombosis, and women with pre-eclampsia have an increase in immediate and lifetime risk of adverse thrombotic events. The mechanisms linking reproductive processes and thromboembolic disease are not well understood. Outwith pregnancy, stimulated tissue plasminogen activator (t-PA) release, platelet activation, circulating endothelial progenitor cell (EPC) number and arterial stiffness are established indicators of vascular pathology and prognosis. The effect of the menstrual cycle, normal and pre-eclamptic pregnancy upon specific areas of vascular health; thrombosis, angiogenesis and arterial stiffness, using the markers described above is the subject of this thesis. The background is presented below.

1.1 THE VASCULAR ENDOTHELIUM

The vascular endothelium is a metabolic organ forming a dynamic interface between blood and tissues. Comprised of a monolayer of cells it is central to the regulation of blood vessel tone and permeability; acts as a non-adhesive surface for leucocytes and platelets and produces important factors in the regulation of fibrinolysis and blood flow.

The term 'endothelial dysfunction' is widely used to imply disturbance of normal homeostatic pathways, predisposing to vasoconstriction, platelet activation and thrombosis. In many clinical studies it is assessed by endothelium-dependent vasomotion [McCarthy *et al*, 1993]. However, solely assessing function by endothelium-dependent vasomotion is restrictive and inaccurate, as altered vasomotion may not reflect other capacities of the endothelial cell, such as t-PA release. For example, both smokers and hypertensive patients have impaired endogenous t-PA release but preserved endothelium-dependent vasomotion [Hrafnkelsdottir *et al*, 1998; Pretorius *et al*, 2002; Hrafnkelsdottir *et al*, 2004b]. Recognising differing states of endothelial cell activation, where some pathways may be up-regulated and others inhibited will aid understanding of the role of the endothelium in different pathologies.

1.1.1 ENDOTHELIAL CELL ACTIVATION

Endothelial cell activation was defined in 1988 as "a quantitative change in the level of expression of certain gene products (proteins) that in turn, endow endothelial cells with new capacities to perform new functions" [Pober, 1988]. Endothelial cell activation encompasses many interlinked responses such as the immunoregulatory cytokine interleukin (IL)-1 stimulating thrombin production. There are five main components of endothelial cell activation: loss of vascular integrity; expression of leucocyte adhesion molecules (P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1); secretion of cytokines (IL-1, IL-6, IL-8, monocyte chemo tactic protein (MCP)); pro-thrombotic changes; and up-regulation of human leucocyte antigen (HLA) molecules (Figure 1).

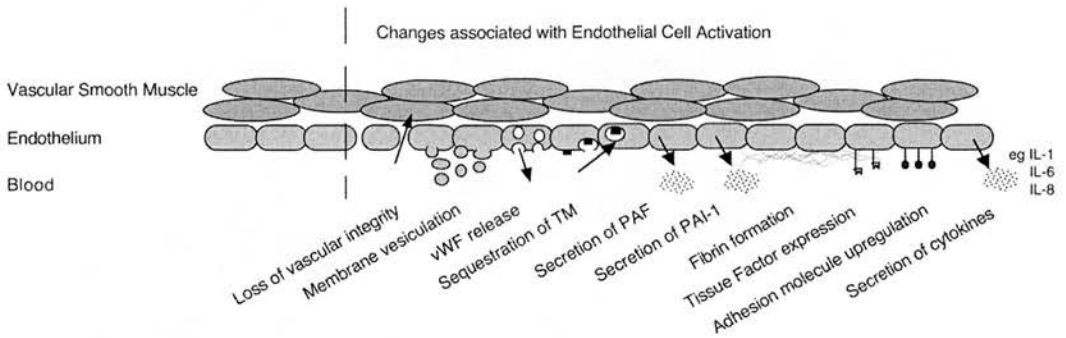

Figure 1. Changes associated with endothelial cell activation.
vWF - von Willebrand factor; TM - thrombomodulin; PAF - platelet activating factor; PAI-1 - plasminogen activator inhibitor type 1; IL - interleukin. Adapted from Hunt BJ, 2002 [Hunt BJ, 2002].

The loss of vascular integrity and expression of leucocyte adhesion molecules results in leucocyte tethering and subsequent migration into underlying tissues. In addition to being a target for cytokine action as a result of infectious and non-infectious inflammatory states, the endothelium is a source of cytokines, particularly IL-1 and IL-6 and the chemo attractants IL-8 and MCP, further potentiating the inflammatory response. The pro-thrombotic effects of endothelial cell activation are due to loss of antithrombotic effects and expression of thrombotic molecules and are described in detail below.

1.1.2 Impaired endothelium-dependent vasomotion

The ability of acetylcholine to induce relaxation of vascular smooth muscle is dependent on endothelial cells, endothelium-dependent relaxation. Damage to, or removal of the endothelium results in impaired relaxation of the vascular smooth muscle to acetylcholine, though it still responds to glyceryl trinitrate. Endothelium-dependent relaxation is in part mediated by nitric oxide, continuous basal release of which from the vascular endothelium maintains resting tone. Impaired endothelium-dependent relaxation is associated with several disease states, including atherosclerosis, hypertension and diabetes and is affected by increasing age and cigarette smoking.

1.2 THROMBOSIS

1.2.1 ENDOGENOUS FIBRINOLYSIS

The endothelium plays a central role in haemostasis, through its regulation of coagulation and fibrinolysis. In a healthy state it produces the anticoagulants thrombomodulin, protein S, tissue factor pathway inhibitor and heparin sulphate proteoglycans as well as inhibitors of platelet aggregation [Jin *et al*, 2005]. Disturbances in normal endothelial function secondary to injury or inflammation can cause it to become pro-coagulant, through down-regulation of its anticoagulant functions; increased secretion of platelet activating factor, von Willebrand factor (vWF) and fibronectin, as well as increased tissue factor expression [Rosenberg and Aird, 1999; van Hinsbergh, 2001]. The role of the endogenous fibrinolytic system is to protect the circulation from intravascular fibrin formation and thrombosis.

Tissue plasminogen activator

Tissue plasminogen activator is a 68kDa serine protease synthesised and released, both constitutively and faculatively by the endothelium. The pathways that regulate release are poorly described, though facultative release is thought to involve G-coupled protein receptors and intracellular calcium signalling. Factors stimulating release include factor Xa and thrombin from the clotting cascade, as well as bradykinin [Emeis, 1992; Brown *et al*, 1997]. The synthesis of t-PA varies with anatomical site, reflecting heterogeneity in endothelial cell function. The capacity of the endothelium to secrete t-PA is large; continuous release can be stimulated for many hours and concentrations in the forearm approach those achieved by systemic

thrombolytic therapy [Witherow *et al*, 2002]. Once released by the endothelium, t-PA catalyses the conversion of plasminogen (which circulates in molar excess) to plasmin, which in turn degredates fibrin to soluble fibrin degredation products. The plasma half-life of t-PA is approximately 5 minutes and it is predominately cleared by the liver [Narita *et al*, 1995].

Little circulating t-PA is functionally active, as the majority is bound to the serine protease inhibitors, mostly plasminogen activator inhibitor type 1 (PAI-1) [Nordenhem and Wiman, 1998]. The proportion of active t-PA in the circulation varies inversely with PAI-1 concentrations, as PAI-1 circulates in molar excess of t-PA and complex formation between the t-PA and PAI-1 is rapid, thus limiting circulating active t-PA (Figure 2).

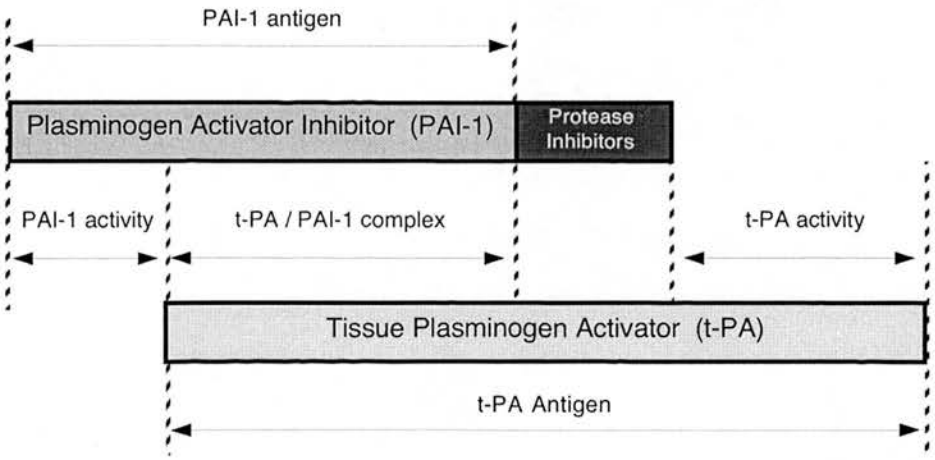

Figure 2. PAI-1 binds most t-PA in plasma. Only unbound fractions are active. Other protease inhibitors include PAI-2, which is produced by the placenta in pregnancy.
PAI-1 - plasminogen activator inhibitor type 1; t-PA - tissue plasminogen activator. Adapted from Oliver *et al*, 2005 [Oliver *et al*, 2005].

To achieve effective thrombolysis, quick local release of t-PA is required. The relative balance between plasma t-PA and PAI-1, and the acute release of t-PA from the endothelium determines the efficacy of endogenous fibrinolysis during intravascular thrombus formation or endothelial injury [Oliver *et al*, 2005]. The local capacity for acute endothelial t-PA release is not reflected by basal plasma t-PA concentrations [Hrafnkelsdottir *et al*, 2004a], which highlights the importance of assessing acute local endothelial t-PA for its pathophysiological relevance.

Clinical relevance

Impaired t-PA release has been demonstrated in the forearm of cigarette smokers and hypertensive subjects who are at increased risk of thrombotic disease [Newby *et al*, 1999; Ridderstrale *et al*, 2006]. In stable coronary heart disease patients, reduced acute t-PA release predicts future cardiovascular events [Robinson *et al*, 2007]. Gender differences exist in acute endothelial t-PA release with middle-aged women releasing more than men [Stauffer *et al*, 2005]. The hypercoaguable state of pregnancy is associated with increased basal plasma concentrations of PAI-1 and PAI-2 [Hellgren, 2003], due in part to their production by the placenta [Vassalli *et al*, 1991]. However, acute endothelial t-PA release has not been fully evaluated in pregnancy.

Assessment of acute endothelial t-PA release

The facultative release of t-PA by the endothelium can be demonstrated in humans *in vivo*, by use of several clinical models using infusions of bradykinin and substance P to stimulate release. Systemic release can be measured following intravenous

infusion of these agents but is not thought to represent local t-PA activity due to the confounding factors of clearance and PAI-1 complex formation. The venous occlusion test, which measures increased concentrations of pooled t-PA in venous plasma in an upper or lower limb has been used but is now surpassed by the combined technique of venous occlusion together with intra-arterial infusions of stimulant drugs. The most common method used in clinical research involves infusions of substance P, bradykinin or desmopressin into the brachial artery and collecting outflowing venous blood from both the infused and non-infused arms. Net release of t-PA can be calculated from the concentration difference in plasma t-PA antigen and activity between arms, the haematocrit (Hct) and forearm blood flow (FBF) and is described in more detail in Chapter 2. This method is highly reproducible and provides an accurate assessment of stimulated t-PA release as well as endothelium-dependent and independent vasomotion [Newby *et al*, 2002].

1.2.2 Interaction of platelets and monocytes

Platelets are critical to haemostasis through clot formation following activation of the coagulation pathway. In addition platelets mediate inflammation through interactions with circulating leucocytes and the endothelium, together with the release of platelet-derived chemokines [Henn *et al*, 1998; Levi *et al*, 2003].

Platelets may either become activated in the circulation by cytokines including IL-1 and IL-6 associated with systemic inflammatory or thrombotic events, or by interactions with activated endothelium [Levi *et al*, 2003]. In the non-activated state the endothelium prevents adhesion of platelets to the vessel wall by release of nitric

oxide, prostacyclin and cyclooxygenase-2 [Jin *et al*, 2005]. As described above, endothelial cell activation results in a pro-thrombotic endothelial phenotype with the release of vWF and the up-regulation of tissue factor and adhesion molecules such as ICAM-1. These changes further promote platelet-endothelial interactions and subsequent platelet activation [Rosenberg and Aird, 1999; van Hinsbergh, 2001]. Platelet-endothelial cell binding and platelet release of IL-1β results in further endothelial cell activation via nuclear factor kappa β (NF-kβ) activation, with subsequent up-regulation of adhesion molecules on the endothelial cell, including ICAM-1 and P-selectin as well as release of MCP-1, IL-6 and 8, vWF and matrix metalloproteinases [Hawrylowicz *et al*, 1991]. Thus platelet adhesion results in a pro-inflammatory endothelium.

When platelets become activated, conformational changes occur in the glycoprotein (GP) αIIbβ3 (GP IIb IIIa) and in the integrin α2β1 resulting in exposure of the fibrinogen and collagen binding sites, respectively. These changes mediate firm adhesion of the platelet to the endothelium. In addition, exposure of the α-granule membrane protein, P-selectin on the platelet surface occurs together with cluster of differentiation (CD)40 ligand (CD40L/CD154) and tumour necrosis factor super family 14 expression [Henn et al, 1998; Michelson, 2006]. The activated platelet binds secreted platelet proteins, thrombospondin and multimerin to its surface and releases inflammatory mediators including IL-1β and platelet factor (PF)4 [van Gils *et al*, 2009].

Adherent platelets recruit monocytes

Adherent, activated platelets on the endothelial monolayer also recruit monocytes. This happens directly between P-selectin expressed on the activated platelet and P-selectin glycoprotein ligand-1 (PSGL-1) constitutively expressed on the monocyte [Kuijper *et al*, 1998]. Interaction of these surface molecules also induces monocyte expression of membrane-activated complex-1 (Mac-1) (CD11b/CD18) which promotes further monocyte to platelet binding through its association with GP1b and junctional adhesion molecule (JAM)-c on platelets [Neumann *et al*, 1999]. The CD40-CD40 ligand dyad also mediates further platelet-monocyte interactions. Indirectly, chemokines released by the activated platelet such as PF4 and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) also recruit monocytes, promoting adhesion to the vessel wall [Lindmark *et al*, 2000].

Circulating activated platelets bind leucocytes

System inflammatory or thrombotic conditions can result in circulating activated platelets, binding to leucocytes, predominately monocytes to form complexes: known as circulating platelet-monocyte aggregates (Figure 3). This interaction is predominately due to P-selectin on platelets, binding to PSGL-1 on monocytes [Sarma *et al*, 2002], with stabilisation occurring through monocyte Mac-1 and platelet GP1b. The formation of platelet-monocyte aggregates depends mostly on platelet activation and is considered to be a sensitive marker of *in vivo* platelet activation [Michelson *et al*, 2001]. Importantly, platelet-monocyte aggregates have multiple functional consequences [van Gils *et al*, 2009].

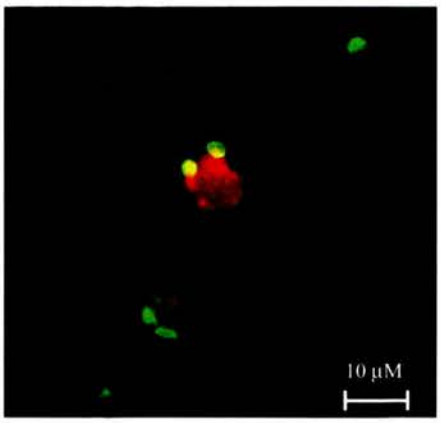

Figure 3. Monocytes form multiple conjugates with platelets. Whole unfragmented platelets (green) are bound to monocytes (red). Unbound platelets are also clearly visible. Reproduced with permission from Sarma *et al,* 2002 [Sarma *et al*, 2002].

Monocyte activation and interaction with the endothelium

Platelet-monocyte aggregates have increased adhesive and transmigrate capacity compared to platelet-free monocytes [van Gils *et al*, 2009]. Binding of platelets to monocytes, through P-selectin-PSGL-1 and also CD40-CD40L, together with the action of platelet-derived chemokines results in monocyte activation, with shedding of L-selectin. Platelet-monocyte interactions also increase NF-kβ activity within the monocyte with subsequent release of pro-inflammatory mediators including tumour necrosis factor (TNF)-α, IL-8, MCP-1, IL-1β and tissue factor [Neumann *et al*, 1997; Li *et al*, 2008]. Finally, increased expression and activity of B_1 and B_2 (Mac-1) integrins increase the adhesive capacity of monocytes which in turn affects their ability to tether to endothelial cells [da Costa Martins *et al*, 2004] and decreases

their rolling velocity, secondary to L-selectin shedding. Together, these result in increased contact of the monocyte with endothelial cells and exposure to chemokines and adhesion molecules. RANTES, IL-8 and MCP-1 released by platelets and endothelial cells promote the arrest of rolling monocytes, and endothelial cell expression of integrins such as ICAM-1 binding to Mac-1 on monocytes cause stable adhesion of the monocyte to the endothelium [Gerszten *et al*, 1999]. Subsequent to stable adhesion on the endothelium, monocytes transmigrate into the sub-endothelial space.

Clinical relevance

Platelet, monocyte and endothelial cell activation are closely linked. Interaction of activated platelets with monocytes, either within the circulation or on the endothelial surface results in monocyte activation. The activated monocyte becomes more adhesive, migratory, pro-inflammatory and pro-coagulant and causes further endothelial, platelet and monocyte activation. Platelet-monocyte aggregates as markers of platelet activation are elevated in vascular pathologies such as myocardial infarction and stroke and are increasingly considered to be a cardiovascular risk factor [Furman *et al*, 1998]. Establishing the effect of reproductive processes on platelet and monocyte activation may help link thromboembolic disease and reproductive processes.

Assessment of platelet- monocyte aggregates, platelet and monocyte activation

The technique of flow cytometry, described in detail in Chapter 2, allows the detection of specific activation-dependent changes on the platelet surface as well as

their interactions with other cells including monocytes. Platelets are extremely susceptible to *ex vivo* activation by sampling and handling methods. The use of flow cytometry allows evaluation of *in vivo* platelet and monocyte activation in whole blood [Michelson *et al*, 2000]. Using this technique, the expression of platelet surface P-selectin, monocyte surface CD40 and CD11b and the formation of circulating platelet-monocyte aggregates can be quantified. These are considered highly sensitive and physiologically relevant markers of platelet [Michelson *et al*, 2001] and monocyte activation [Shi and Simon, 2006].

1.3 ANGIOGENESIS

The discovery of endothelial progenitor cells has generated considerable interest in the field of vascular biology. These cells arise from a population of circulating mononuclear cells and have the capacity to form new blood vessels and contribute to vascular repair. Circulating EPC numbers are reduced in patients with cardiovascular risk factors and in the presence of endothelial dysfunction, but are increased in response to ischaemia, oestrogens and drug therapy. They have been studied in pathologies from cardiovascular and renal disease to rheumatoid arthritis and pre-eclampsia.

1.3.1 CHARACTERISATION OF CIRCULATING EPCS

Endothelial progenitor cells are characterised by their expression of both haematopoietic and mature endothelial cell antigens, and by their ability to proliferate, migrate and differentiate into mature cell types. Asahara *et al* exploited

two antigens shared by endothelial cells and haematopoietic stem cells (HSCs) to isolate putative EPCs from peripheral blood [Asahara *et al* 1997]. CD34 is expressed by most mature endothelial cells [Fina *et al*, 1990] as well as all HSCs but is lost by haematopoietic cells as they differentiate [Civin *et al*, 1984]. Kinase insert domain receptor (KDR), the extracellular domain of vascular endothelial growth factor (VEGF) receptor [Shalaby *et al*, 1995], is also expressed by both early HSCs and endothelial cells but is lost on haematopoietic cell differentiation [Matthews *et al*, 1991]. CD34 positive ($^{+}$) and KDR^{+} cells isolated from peripheral blood leucocytes form vascular structures *in vitro* and incorporate into the vessel wall in experimental models of neovascularisation [Asahara *et al*, 1997].

Co-expression of CD34 and KDR has been used in a number of experimental and clinical studies to identify circulating EPCs. No surface marker unique to endothelial progenitors has been identified and so it remains difficult to distinguish EPCs from mature endothelial cells that have been swept into the circulation and haematopoietic cells. CD133 is expressed by haematopoietic cells, but not by mature endothelial cells. Identification of CD133, KDR and CD34 co-expression may differentiate between circulating mature and progenitor endothelial cells [Peichev *et al*, 2000]. The rarity of EPCs in peripheral blood (100-200 cells mL^{-1}) has made their study difficult.

Alternative methods have been described for the characterisation and quantification of EPCs based on the culture of endothelial cells from circulating mononuclear cells. A number of functional assays have been reported, most involving the isolation of

peripheral blood mononuclear cells by density centrifugation of blood and subsequent culture on fibronectin coated plates. After 5 to 7 days in culture, adherent colonies are seen, where spindle shaped cells emerge from a cluster of round cells (colony forming unit-endothelial progenitor cells (CFU-EPCs)). These adherent cells display a variety of endothelial-like properties including the uptake of acetylated low-density lipoprotein (AcLDL) and staining with a lectin of Ulex europaeus (UEA-1), (specific for endothelial cells in a variety of tissues binding to the carbohydrate moiety α-L-fucose [Stephenson *et al*, 1986]).

Studies addressing the origin of EPCs have demonstrated that monocytes express endothelial lineage markers such as KDR and can differentiate into endothelial cells [Schmeisser *et al*, 2001]. Rehman *et al*, found that the majority of CFU-EPCs expressed monocyte markers such as CD14, Mac-1, and CD11c, suggesting that peripheral blood EPCs are derived from monocyte-like cells [Rehman *et al*, 2003]. The concept that functional endothelial cells may originate from a $CD14^+$ progenitor is supported by reports that mature endothelial cells from human umbilical vein express CD14 [Jersmann *et al*, 2001] and that isolated $CD14^+$ cells can improve neovascularisation after mouse hind limb ischaemia [Urbich *et al*, 2003]. Figure 4 outlines two potential ways that endothelial cells might arise from HSCs via myeloid or endothelial progenitor subtypes.

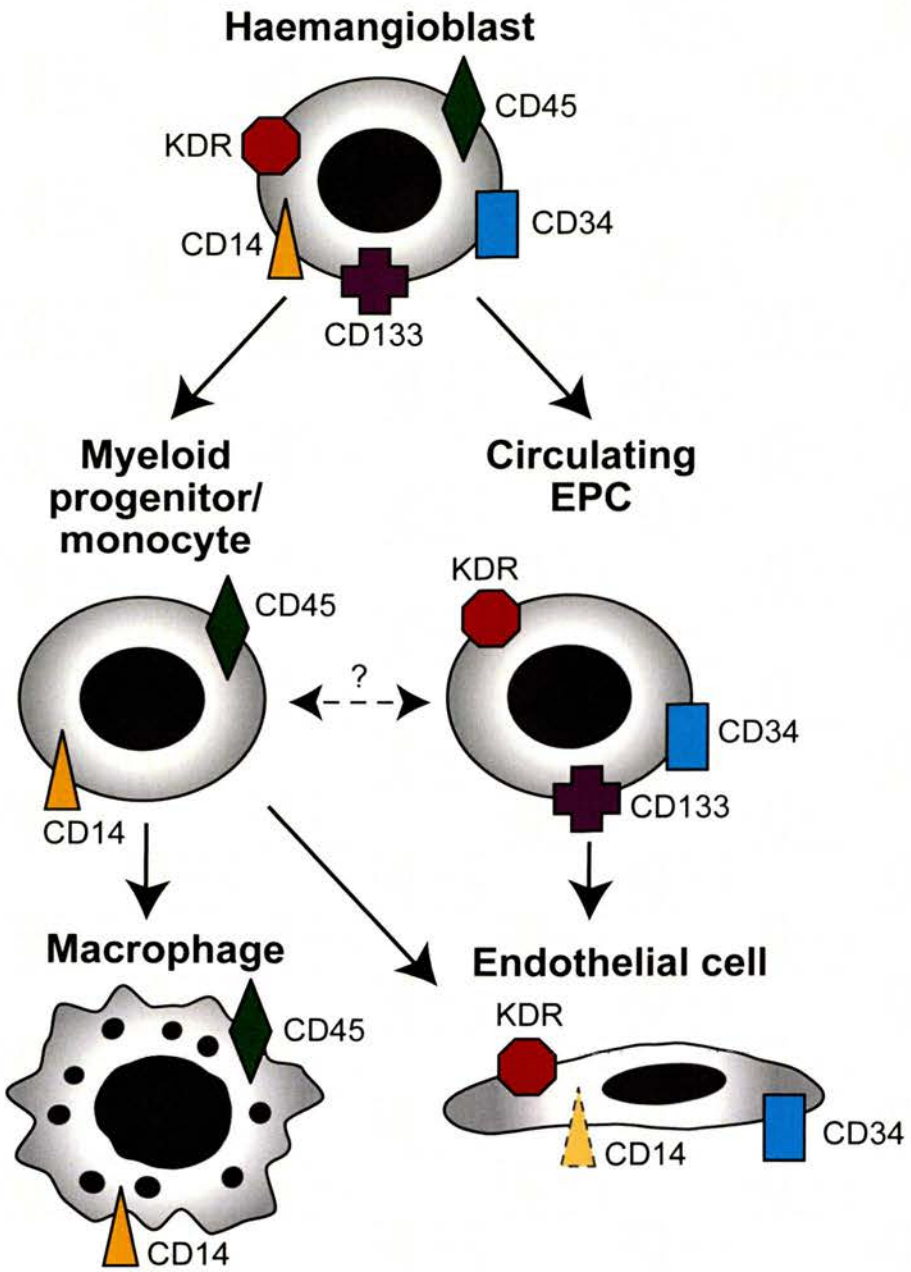

Figure 4. Possible pathways by which endothelial cells may arise from haematopoietic stem cells. Haemangioblasts give rise to circulating haematopoetic stem cells of myeloid or endothelial progenitor subtypes, with endothelial cells potentially derived from either pathway.
KDR - Kinase insert domain receptor; CD - cluster of differentiation; EPC - endothelial progenitor cell. Reproduced with permission from Robb *et al*, 2007 [Robb *et al*, 2007].

In this emerging field, many different methods have been used to characterise and quantify putative endothelial progenitors, preventing straightforward comparison. Reduced levels of both phenotypic [Schmidt-Lucke *et al*, 2005] and functional EPCs [Werner *et al*, 2005] (Table 1) predict adverse outcome in patients with coronary artery disease. Whilst questions remain as to the origin and phenotype of EPCs, evidence that they contribute to vascular repair and neoangiogenesis in animal models is compelling.

TABLE 1 CHARACTERISTICS OF ENDOTHELIAL PROGENITOR CELLS

Phenotypic	• Cell-surface expression of haematopoietic stem-cell (CD34, CD133) and endothelial cell antigens (KDR)
Functional	• Produce characteristic colonies and tubular structures on fibronectin *in vitro* • Migrate and incorporate into areas of vascular damage *in vivo* • Differentiate into mature endothelial cells

CD - cluster of differentiation; KDR - kinase insert domain receptor.

1.3.2 MOBILISATION AND DIFFERENTIATION OF EPCS

Integrity of the vascular endothelium is a dynamic equilibrium between endothelial degeneration and repair [Karter *et al*, 2004]. The concept of a pool of endothelial progenitors in the bone marrow, capable of moving to effect angiogenesis or vascular repair in response to ischaemia or vascular injury is supported by both *in vitro* and *in vivo* studies. In a mouse model, Asahara *et al* demonstrated differentiation of donor bone marrow cells into endothelial cells and their subsequent incorporation into the

vasculature during processes such as ovulation, wound healing, recovery from hind limb ischaemia and neoplasia [Asahara *et al*, 1999].

The postulated factors responsible for mobilisation of EPCs from the bone marrow are the subject of an intense search. Such a factor might form a therapeutic strategy to enhance vascular repair. EPCs are released in the context of acute ischaemic injury, such as myocardial infarction [Leone *et al*, 2005; Massa *et al*, 2005] and following vascular injury as a consequence of coronary artery bypass grafting [Gill *et al*, 2001]. Vascular endothelial growth factor and stromal cell-derived factor-1 (SDF-1), both released from ischaemic tissue, are thought to be important factors in the mobilisation of EPCs. Other cytokines such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) mobilise both haematological progenitors and EPCs. These have been used for many years to harvest progenitors for autologous bone marrow transplantation in the context of haematological malignancy. However, they also induce a pro-inflammatory state that may limit their therapeutic potential.

Mechanisms of EPC mobilisation in conditions other than ischaemia may be more relevant to understanding the role of EPCs in healthy women. The effect of oestrogens in maintaining endothelial function may be related to EPC mobilisation and enhanced vascular repair. In a rat carotid injury model, exogenous oestradiol accelerates re-endothelialisation and attenuates medial thickening via mobilisation and proliferation of bone marrow-derived EPCs. This response was absent in endothelial nitric oxide synthase knock out animals [Iwakura *et al*, 2003]. Recent

mouse studies with oestrogen receptor (ER) α and β knockout animals demonstrate roles for both receptors in EPC-mediated neovascularisation in response to ischaemia. In addition, ERα messenger ribonucleic acid (RNA) expression was higher than ERβ messenger RNA expression in EPCs. Vascular endothelial growth factor expression was significantly down regulated on EPCs from ERα knockout mice compared with EPCs from wild type animals [Hamada *et al*, 2006].

1.3.3 Clinical relevance

Reduced circulating EPC levels are observed when established cardiovascular risk factors are present suggesting a role for EPCs in the maintenance of endothelial function. Decreased numbers of EPCs have been demonstrated in cigarette smokers [Vasa *et al*, 2001] and in patients with diabetes mellitus [Tepper *et al*, 2002] or rheumatoid arthritis [Grisar *et al*, 2005]. Hill *et al* [2003] observed a strong correlation between the Framingham cardiovascular risk score (which uses cardiovascular risk factors to predict future risk of coronary artery disease [Wilson *et al*, 1987]) and circulating EPCs numbers. Endothelial progenitor cell numbers predicted systemic endothelial function more accurately than the Framingham risk score [Hill *et al*, 2003]. Furthermore, lower levels of EPCs are associated with adverse outcome in patients with coronary artery disease [Schmidt-Lucke *et al*, 2005; Werner *et al*, 2005]. As described, EPC mobilisation and function is affected by inflammation, hormonal regulation and endothelial function. These factors are pivotal to many reproductive processes, including menstruation, healthy pregnancy and complications of pregnancy such as pre-eclampsia.

1.3.4 ASSESSMENT OF CIRCULATING EPCS

Due to a lack of consensus on the definitive EPC phenotype a variety of surface markers are in use to assess this progenitor population. Increasingly EPCs are quantified by surface expression of phenotypic markers ($CD34^+CD133^+KDR^+$) or their ability to form colonies within an endothelial progenitor cell assay [Hill *et al*, 2003]. Relatively few clinical studies have either used both approaches or compared EPC phenotype and function. Both methods are described in detail in Chapter 2.

1.4 ARTERIAL COMPLIANCE AND STIFFNESS

The compliance of a vessel is the amount by which it will increase in volume for a given increase in distending pressure and is determined by the elastic properties of the vessel wall. Distensibility is the proportionate increase in volume per unit increase in distending pressure.

COMPLIANCE = $\Delta V / \Delta P$

Where ΔV is the increase in volume and ΔP the increase in pressure.

DISTENSIBILITY = $[\Delta V/V] / \Delta P$

As compliance depends on the diameter of the vessel, distensibility is more closely related to the elastic properties of the vessel. A larger artery may have greater compliance than a smaller artery due to its larger diameter, even though its intrinsic elasticity may be lower. The term 'arterial stiffness' is often used to represent the

inverse of distensibility when describing the reduction in compliance in a vessel, which dilates very little even when exposed to a large distending pressure.

Vascular compliance has important influences on circulatory haemodynamics. The compliance of the aorta and large arteries accommodates blood ejected from the left ventricle in systole, reducing after load and preventing excessive rises in systolic pressure. In diastole, when distending pressure falls, aortic contraction ensures increased flow to the peripheral and coronary circulations. A poorly compliant or 'stiff' vessel will cause excessive rise in blood pressure with systole and a precipitous fall in diastole. Thus compliance has effects on systemic haemodynamics in systole, and diastole but is also important for its transmission of pressure throughout the vascular tree. When a forward pressure wave propagates through the vascular tree, it arrives at peripheral arteries at a later time during systole. In addition this pressure wave is reflected at various points, mostly by small arteries resulting in reflected waves travelling back towards the heart. The total pressure in the vasculature at any point is the sum of the forward and reflected waves. In a compliant aorta, reflected waves arrive back during diastole with the effect of increasing early diastolic pressure which is thought to be favourable, especially with regard to coronary perfusion. A poorly compliant or 'stiff' aorta will result in faster travel of forward and reflected wave, with a greater proportion of reflected waves arriving in systole, augmenting systolic blood pressure with detrimental effects [Laurent *et al*, 2006; Hunt, 2002].

1.4.1 ARTERIAL WALL STRUCTURE

The intrinsic elasticity of the artery wall is determined by the individual components, endothelial, smooth muscle and fibroblast cells within extra cellular matrix (ECM). The endothelium releases locally active mediators such as nitric oxide, which have immediate vasoactive properties and longer lasting trophic effects on vascular smooth muscle cells (VSMCs). Vascular smooth muscle cells actively control wall tension and also synthesise major structural components of the vascular wall. The ECM comprises more than 60% of the intimal volume and is made up of collagen and elastin, embedded in glycoproteins and proteoglycans. The ECM is increasingly recognised as a dynamic structure, acting as a store for growth factors and containing the enzymes, matrix metalloproteinases. Both are thought to affect cellular growth and proliferation with resulting reorganisation of the ECM [Dollery *et al*, 1995; Taipale and Keski-Oja, 1997]. In muscular arteries, compliance is greatly affected by smooth muscle tone, with a decrease in tone associated with an increase in compliance. In larger elastic arteries such as the aorta, compliance is less dependent on vascular smooth muscle tone than the intrinsic elasticity of the ECM.

1.4.2 CLINICAL RELEVANCE

Increased arterial stiffness is an independent predictor of organ damage and cardiovascular outcomes [Saba *et al*, 1993; Booth *et al*, 2004; Weber *et al*, 2005]. Within normotensive pregnancy, aortic stiffness is inversely associated with birth weight, more than mean arterial pressure, suggesting that increased arterial compliance may be a better representation of maternal adaptation to pregnancy than blood pressure [Elvan-Taspinar *et al*, 2005]. Arterial stiffness is widely assessed in

observational studies to further understand the mechanisms underlying haemodynamic changes in clinical conditions and increasingly used to examine responses to pharmacocological and non-pharmacocological interventions.

1.4.3 Assessment of arterial compliance and stiffness

Arterial compliance can be determined in several ways; high-resolution ultrasound can be used to measure changes in vessel diameter throughout the cardiac cycle, together with tonometry to determine pressure to calculate the compliance of segments of the arterial tree. Two other methods, pulse wave analysis (PWA) and the gold standard measurement, pulse wave velocity (PWV) and are in common use and are described in detail in Chapter 2.

1.5 THE MENSTRUAL CYCLE

The normal regular menstrual cycle is about 26 to 35 days in length. It is controlled by the hypothalamus and pituitary glands, and is divided simplistically into two phases by the occurrence mid-cycle of ovulation. The first half of the cycle is the follicular phase and the second half the luteal phase (Figure 5). The start of the cycle is defined by menstruation, the process of shedding of the functional layer of the endometrium. This has been described as an inflammatory event with local up-regulation of cytokines, together with recruitment of leucocytes [Critchley *et al*, 2001]. At this point, the early follicular phase, systemic levels of both oestradiol and progesterone are low. The mid and late follicular phases are characterised by gradually increasing levels of oestrogen produced by the developing follicle. This is

followed by the lutenising hormone (LH) surge and ovulation. The early luteal phase is characterised by slightly lower concentrations of oestrogen and high levels of progesterone. In the absence of pregnancy, progesterone levels fall following the demise of the corpus luteum and menstruation follows.

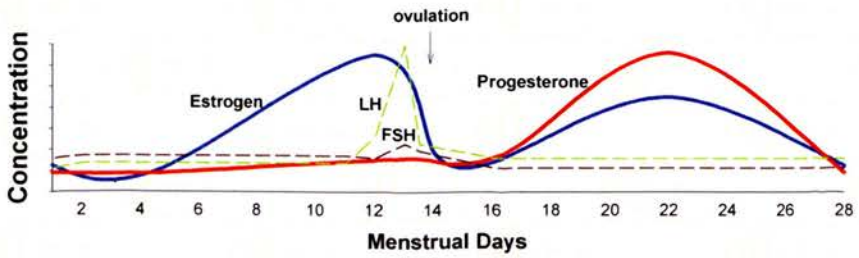

Figure 5. Cyclical variation in ovarian hormones during the menstrual cycle.
LH - lutenising hormone; FSH - follicle stimulating hormone. Adapted from Simmen *et al* 2006 [Simmen *et al*, 2006].

1.5.1 THROMBOSIS

Although rare in pre-menopausal women, arterial thrombotic events do occur in some women and incidence appears to be affected by menstrual cycle phase. During menstruation, when circulating oestradiol concentrations are lowest, the incidence of effort and variant angina are increased [Lloyd *et al*, 2000; Kawano *et al*, 2001] as is the incidence of myocardial infarction [Mukamal *et al*, 2002].

Little data exists for the effect of the menstrual cycle, upon endogenous fibrinolysis or platelet activation, in the absence of exogenous hormones. Systemic levels of t-PA and PAI-1 have been measured throughout the menstrual cycle and found not to vary [Koh *et al*, 2005]. The capacity of the endothelium to release t-PA has been assessed at different stages of the cycle using a venous occlusion test. The late luteal phase

and the menstrual phase of the cycle were both associated with a significant fall in fibrinolytic capacity compared to the other phases of the cycle [Siegbahn *et al*, 1989]. Both the above studies measured platelet function *in vitro* and found no cyclical variation [Siegbahn *et al*, 1989; Koh *et al*, 2005]. Both venous occlusion to examine fibrinolytic capacity and *in vitro* assays of platelet activation have been surpassed by the more sensitive and reproducible tests of forearm venous plethysmography and flow cytometry of whole blood described above. At the outset of this project, neither had been used to examine the effects of the menstrual cycle.

1.5.2 Angiogenesis

Growth of endometrial vasculature in preparation for implantation begins in the proliferative phase and continues in the secretory phase of the menstrual cycle. This physiological angiogenesis is thought to occur primarily through elongation and intussusception of existing small vessels [Gargett and Rogers, 2001]. Sprouting is important in placentation and is also a feature of angiogenesis associated with pathology such as ischaemia [Reynolds and Redmer, 2001] (Figure 6). Asahara *et al* have implicated a role for EPCs in physiological endometrial angiogenesis where EPCs were demonstrated within vasculature and stroma of the endometrium and myometrium after induced ovulation in mice [Asahara *et al*, 1999]. These animal studies are supported by clinical studies in women demonstrating that stromal and endothelial endometrial cells can originate from donor-derived bone marrow cells [Risau and Flamme, 1995; Taylor, 2004].

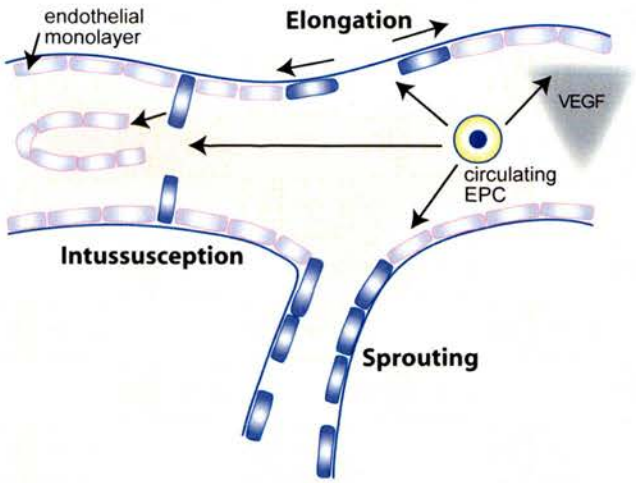

Figure 6. Proposed mechanisms by which endothelial progenitor cells (EPCs) may contribute to angiogenesis in human endometrium. Mature resident endothelial cells (purple) and proliferating resident mature endothelial cells (blue). Circulating EPCs (yellow) may incorporate into endothelial monolayer at points of elongation and intussusception, or proliferate to form new micro vessels (sprouting). Circulating EPCs may have a supportive paracrine effect on adjacent mature endothelial cells, through the release of angiogenic factors including vascular endothelial growth factor (VEGF). EPCs release VEGF both in *in vitro* and in *in vivo* murine studies [Urbich *et al*, 2005]. Reproduced with permission from [Robb *et al*, 2007].

Local factors including oestradiol, TNF-α, IL-6, VEGF and ICAM-1 are implicated in the regulation of endometrial angiogenesis [Jabbour *et al*, 2006]. Many of these factors also appear to be involved in EPC mobilisation and trafficking in mouse models [Iwakura *et al*, 2003; Hamada *et al*, 2006; Iwakura *et al*, 2006] and human studies [Naiyer *et al*, 1999; Kalka *et al*, 2000]. At the commencement of this research, no studies had assessed the effect of the menstrual cycle on circulating levels of EPCs in human subjects.

1.5.3 ARTERIAL COMPLIANCE AND STIFFNESS

Oestrogen and progesterone both have effects on arterial structure and function [Natoli *et al*, 2005]. In the pre-pubertal and post-menopausal years when female sex steroids are low, women have stiffer arteries than age-matched men [Waddell *et al*,

2001; Ahimastos *et al*, 2003]. During the menstrual cycle, female sex steroids fluctuate cyclically, though the available data on the effect of these fluctuations on arterial stiffness is conflicting. Previous studies demonstrate either no change [Willekes *et al*, 1997] or an increase in compliance in the ovulatory phase compared to the follicular and luteal phase [Giannattasio *et al*, 1999; Williams *et al*, 2001]. These studies are not easily comparable because of differing methodologies employed, sample population characteristics, and timing of sampling. Therefore, the aim of the studies undertaken in this thesis was to address these issues by measuring arterial stiffness with a sensitive, reproducible technique in a well-characterised population at clearly defined time-points.

1.6 HEALTHY PREGNANCY

Pregnancy necessitates cardiovascular adaptation to sustain the developing foetus. Initially, peripheral vascular resistance falls resulting in an increase in cardiac output and plasma volume expands [Ginsburg and Duncan, 1967; Ueland *et al*, 1969; Pirani *et al*, 1973]. In normal pregnancy, there is enhanced endothelial function and reactivity with greater production of endothelium-derived vasodilators [Goodman *et al*, 1982; Williams *et al*, 1997; Anumba *et al*, 1999a]. Flow-mediated dilatation of the brachial artery increases with the gestational week suggesting maintenance of endothelial vascular function [Dorup *et al*, 1999].

1.6.1 Inflammatory state

Endothelium-dependent vasodilatation is preserved in spite of the systemic inflammatory response associated with normal pregnancy. There is increased production of pro-inflammatory cytokines, including IL-6, IL-12, and TNF-α with associated leucocytosis. The neutrophil count rises steadily throughout gestation, to peak at term [Austgulen *et al*, 1994; Rebelo *et al*, 1995; Melczer *et al*, 2003; Sacks *et al*, 2003]. As well as being a pro-inflammatory state, normal pregnancy is associated with increased insulin resistance and hyperlipidaemia, controlled by hormonal changes. Pregnancy might be considered a 'stress' test for the maternal vascular endothelium [Sattar and Greer, 2002].

1.6.2 Thrombosis

The hypercoagulable state of pregnancy helps protect women from potentially catastrophic haemorrhage during placentation and the post-partum period [Hellgren, 2003]. However, this predisposes women to thrombosis and thromboembolism, the leading causes of maternal mortality in the United Kingdom [Lewis 2005]. The risks of venous and arterial thromboembolism are both increased 4-fold in pregnancy. Moreover in the post-partum period, these risks are up to 20-fold higher [James *et al*, 2007]. In addition, many complications of pregnancy, such as placental abruption, pre-eclampsia and foetal growth restriction are either caused or exacerbated by the hypercoagulable state [Gilabert *et al*, 1995].

The pro-thrombotic state of pregnancy has been attributed to marked alterations in the coagulation and fibrinolytic system, with increased plasma concentrations of

coagulation factors VII, VIII, X, vWF and fibrinogen as well as reductions in plasma protein S concentrations. Free, active unbound protein S concentrations are reduced secondary to increased levels of its binding protein, complement component C4b [Hellgren, 2003]. During pregnancy, changes in the fibrinolytic system include increased basal plasma concentrations of both t-PA and its principal inhibitor, PAI-1 [Hellgren, 2003]. Plasma levels of PAI-2, also produced by the placenta, rise dramatically in the third trimester. Tissue plasminogen activator activity during pregnancy has been shown to be reduced compared to non-pregnant women, using the venous occlusion test [Stegnar *et al*, 1993], though the authors acknowledged in a later publication that the technique 'needs improvement in order to be potentially clinically useful'. The pro-thrombotic state begins with conception, aggravated further in late gestation as the gravid uterus partially compresses the inferior vena cava, causing venous stasis in the lower limbs. Disturbances in coagulation and fibrinolysis persist post-partum and make take up to 8 weeks to return to pre-conception values [James *et al*, 2007].

1.6.3 Angiogenesis

The uterine vasculature undergoes dramatic remodelling during pregnancy. In addition to vasodilatation of the uterine artery, remodelling of maternal spiral arteries provides a large vascular bed perfusing the placental intervillous space with maternal blood [Brosens *et al*, 1967]. During placentation, this remodelling is mediated by interstitial and endovascular trophoblast invading maternal vessels [Pijnenborg *et al*, 2006]. During the wave of trophoblast invasion, maternal spiral artery endothelium is extensively damaged and then repaired. This results in a fresh layer of endothelium

[Pijnenborg *et al*, 2006]. The repair is thought to be effected by local endothelial cells though it is plausible that circulating EPCs are involved.

At the commencement of this research, circulating EPCs in normal human pregnancy had been examined in two cross-sectional studies. Different methods had been used to characterise and quantify putative endothelial progenitors, preventing straightforward comparisons between the studies. The first, by Sugawara *et al* [2005a] studied circulating EPCs in the peripheral blood of twenty pregnant women. They observed greater numbers of CFU-EPCs at greater gestational age which also correlated with serum oestradiol concentrations [Sugawara *et al*, 2005a].

Oestrogens are known to have vasculoprotective effects, in part due to increasing production of nitric oxide and by decreasing reactive oxygen species [Mendelsohn and Karas, 1999]. Oestrogens also mobilise EPCs from the bone marrow *in vivo*. They inhibit the senescence of EPCs and stimulate VEGF production *in vitro* [Strehlow *et al*, 2003; Imanishi *et al*, 2005a]. Mobilisation of EPCs may be an important mechanism by which oestrogens protect the vascular endothelium during pregnancy. The second study by Gussin *et al* [2002] supported this hypothesis. They cultured peripheral blood mononuclear cells from non-pregnant and pregnant women. Early outgrowth endothelial cells were formed from both groups. Late outgrowth cells, which have a higher proliferative potential, were only formed by the cells from pregnant women. The authors initially hypothesised that these cells were of foetal origin and their original intention was to optimise the culture of foetal cells. To identify foetal cells, they stained for X and Y-chromosomes and discovered that

none of the colonies contained foetal cells. They concluded that endothelial cells were of maternal origin and that pregnancy is associated with mobilisation of EPCs into the circulation [Gussin *et al*, 2002]. Prospective studies following women through gestation would provide more information about EPCs in pregnancy.

1.6.4 Arterial compliance and stiffness

Successful pregnancy relies on adequate cardiovascular adaptation. The initial effect of pregnancy increasing arterial compliance is well documented in both animal and human studies [Hart *et al*, 1986; Poppas *et al*, 1997; Slangen *et al*, 1997; Edouard *et al*, 1998; Smith *et al*, 2004; Mersich *et al*, 2005]. This increased arterial compliance may aid adaptation to the increased cardiac output by preventing excessive rise in systolic blood pressure.

This altered compliance is likely to be driven by altered endothelial function and vascular smooth muscle tone, but in large arteries such as the aorta, by remodelling. Dramatic remodelling of the uteroplacental circulation in pregnancy has been intensively studied and described. Matrix metalloproteinases have a role in both spiral artery remodelling and pathological remodelling of the cardiovascular system. However the remodelling or restructuring of larger arteries in pregnancy has not been studied to the same extent. Information that exists is largely confined to animal studies although the increase in aortic diameter in pregnant women and further increases with parity suggest that pregnancy causes intrinsic modifications of the arterial wall [Hart *et al*, 1986; Easterling *et al*, 1991].

1.7 PRE-ECLAMPSIA

Pre-eclampsia, occurring in 2 to 7% of first pregnancies with 20 to 25% recurrence is the most common medical complication of pregnancy. The disease, characterised by hypertension and proteinuria, is a significant cause of maternal and perinatal morbidity and mortality worldwide [Stone *et al*, 1994; Hauth *et al*, 2000]. Epidemiological data suggest an increased maternal risk of hypertension, coronary and cerebrovascular disease in later life [Wilson *et al*, 2003].

1.7.1 PATHOGENESIS

Hypertension and proteinuria in pre-eclampsia are measurable endpoints of the disease but despite recent advances, the pathogenesis of pre-eclampsia still remains unclear. Predisposing risk factors for the development of pre-eclampsia include genetic predisposition as well as pre-existing medical complications such as obesity, chronic hypertension and diabetes mellitus. Several theories on the pathogenesis of pre-eclampsia exist and the role of the placenta is pivotal. Deficient placentation (decreased trophoblast invasion and subsequent spiral artery remodelling) is seen histologically in most women with pre-eclampsia [Brosens *et al*, 1972; Pijnenborg *et al*, 1991]. In addition, pre-eclampsia is associated with reduced vascular integrity, endothelial cell activation, impaired endothelium-dependent vasodilatation and activation of the coagulation system.

Two main theories have been proposed:

1) The two-stage model of pre-eclampsia: reduced placental blood flow, secondary to abnormal placentation or pre-existing maternal microvascular disease results in a poorly perfused placenta. The placenta then releases a circulating factor or factors into the circulation, which target the maternal endothelium leading to the endothelial cell activation and impaired endothelium-dependent vasomotion [Roberts and Lain, 2002].

2) The continuum theory of inflammatory response to trophoblast debris: in this theory, pre-eclampsia is an exaggeration of the inflammatory response characteristic of normal pregnancy. The exaggerated response is thought to be secondary to increased trophoblast debris from a poorly perfused or larger placenta or due to increased maternal susceptibility [Redman and Sargent, 2000].

1.7.2 ENDOTHELIAL CELL ACTIVATION

Vascular cellular adhesion molecule-1, ICAM-1, E-selectin, endothelin-1, cellular fibronectin and soluble markers of endothelial dysfunction are raised in the blood of women with pre-eclampsia. Some are evident before the clinical features of the disease [Taylor *et al*, 1991; Schiff *et al*, 1992; Kraayenbrink *et al*, 1993; Higgins *et al*, 1998; Bretelle *et al*, 2001]. Other markers of endothelial cell activation including asymmetric dimethylarginine (an endogenous inhibitor of nitric oxide synthesis) [Savvidou *et al*, 2003] also rise before clinical symptoms appear.

1.7.3 Impaired endothelium-dependent vasodilatation

Despite the pathogenesis being still under debate, resistance arteries from women with pre-eclampsia exhibit impaired endothelium-dependent vasodilatation [McCarthy *et al*, 1993]. Women with pre-eclampsia are more likely to have impaired uterine artery doppler waveforms [Campbell *et al*, 1983] and reduced flow-mediated dilatation of the brachial artery at 23 to 25 weeks gestation, suggesting that alterations in endothelial function precede pre-eclampsia [Savvidou *et al*, 2003]. Impaired endothelium-dependent vasodilatation persists beyond pregnancy in women who have had pre-eclampsia [Lampinen *et al*, 2006].

1.7.4 Circulating anti-angiogenic factors

The link between deficient placentation and endothelial cell activation in pre-eclampsia has been attributed to a circulating factor. It has previously been demonstrated that serum from women with pre-eclampsia is cytotoxic to human umbilical vein endothelial cells (HUVECs) in culture and that plasma from women with pre-eclampsia impairs the normal vasodilatory responses of myometrial vessels to bradykinin [Rodgers *et al*, 1988; Hayman *et al*, 2001]. Despite intensive investigation, the factor or factors are still elusive. Candidates include circulating anti-angiogenic factors such as endoglin and soluble fms-like tyrosine kinase 1 and syncytiotrophoblast micro fragments [Levine *et al*, 2006].

1.7.5 THROMBOSIS

Thrombosis and pre-eclampsia are intertwined. In pre-eclampsia, there is extension of the hypercoagulable state of pregnancy with intravascular coagulation. Women with pre-eclampsia are at an increased risk of arterial and venous thromboses [Kobayashi *et al*, 1999]. In addition, women with a history of hypercoaguability, manifested by previous thromboembolism are at increased risk of pre-eclampsia [Robertson *et al*, 2006]. Both PAI-1 and t-PA are elevated in the blood of pregnant women who develop pre-eclampsia before clinical symptoms appear, with t-PA correlating with the degree of proteinuria [Belo *et al*, 2002; Roes *et al*, 2002]. It is often assumed that pre-eclampsia is accompanied by platelet activation. However, the existing literature is controversial with studies demonstrating that platelet activation may be increased [Konijnenberg *et al*, 1997; Harlow *et al*, 2002; Bagamery *et al*, 2005] or unchanged [Holthe *et al*, 2005] compared to normal pregnancy.

Within the placenta, increased synthesis of PAI-1 [Estelles *et al*, 1998] is associated with aberrant fibrin deposition and the development of vaso-occlusive lesions within the placenta [Ma *et al*, 2002]. Acute atherosis in the placental bed, with lipid-laden foam cells is similar to atherosclerosis found elsewhere in the vascular tree, out with pregnancy. Thrombotic risks are not confined to pregnancy. Women with a history of pre-eclampsia have increased risks of venous and arterial thromboembolism in later life compared to women with uncomplicated pregnancies [van Walraven *et al*, 2003; Ray *et al*, 2005].

1.7.6 ANGIOGENESIS

Pre-existing conditions associated with endothelial dysfunction, such as hypertension, renal disease, and diabetes, increase the risk of developing pre-eclampsia. Although there are extensive studies reporting decreased levels of EPCs or abnormal function of EPCs in men and non-pregnant women with these conditions, there are few available data about EPCs in pre-eclampsia. Sugawara *et al* [2005b] demonstrated decreased numbers of CFU-EPCs and increased senescence of EPCs in patients with pre-eclampsia compared to gestationally matched controls. [Sugawara *et al*, 2005b]. It is likely that EPC function is more important than quantity, and ideally a subsequent study of EPCs in pre-eclampsia would assess number and function prospectively prior to the onset of disease.

1.7.7 ARTERIAL COMPLIANCE AND STIFFNESS

Current clinical practice relies on the measurement of brachial blood pressure as a screening and diagnostic test for pre-eclampsia. However, because of the amplification of pulse pressure between central and peripheral arteries, peripheral blood pressure is not an accurate reflection of central aortic pressure [Wilkinson *et al*, 2001a]. Central pressures are more closely associated with intermediate cardiovascular outcomes than brachial pressures in non-pregnant and pregnant populations [Jondeau *et al*, 1999; Roman *et al*, 2007]. The techniques of pulse wave analysis mentioned briefly above enable the non-invasive determination of central arterial pressure and stiffness from the radial artery waveform. Use of this technique in women with pre-eclampsia may further help our understanding of the disease and

if central pressures and changes in arterial stiffness could be detected before a rise in brachial blood pressure, the technique may have use as a screening tool.

1.8 SUMMARY

This thesis concerns aspects of vascular function in the menstrual cycle, normal and pre-eclamptic pregnancies. By assessing different areas of vascular function; thrombosis, angiogenesis and arterial stiffness, the effect of varying reproductive states on the vasculature can be further understood. Stimulated t-PA release, platelet activation, circulating EPC number and arterial stiffness are all useful indicators for assessing these areas of vascular function. The above overview of these markers, together with their pathophysiological and clinical relevance provides the background to the following hypotheses.

1.9 HYPOTHESES AND AIMS

1.9.1 THROMBOSIS

Endogenous fibrinolysis

It was hypothesised that the pro-thrombotic state of pregnancy would be reflected in impaired capacity of the endothelium to release t-PA, in response to stimulation with bradykinin (Chapter 3).

The aim of this research was to compare endogenous fibrinolysis between pregnant women and non-pregnant control women using the sensitive technique of forearm occlusion venous plethysmography.

Platelet and monocyte activation

It was hypothesised that menstruation as well as the pro-thrombotic and inflammatory state of pregnancy would be reflected in increased *in vivo* platelet and monocyte activation and that this would be further increased in pre-eclampsia (Chapter 4).

The aim of this research was to make serial measurements of platelet and monocyte activation, throughout the normal menstrual cycle, healthy pregnancy, and pregnancy affected by pre-eclampsia using both cellular (platelet-monocyte aggregates, platelet surface P-selectin expression, monocyte CD40 and CD11b expression) and soluble (soluble P-selectin and soluble CD40L) markers of activation.

1.9.2 Angiogenesis

It was hypothesised that EPCs are mobilised during inflammatory reproductive processes involving endothelial repair, specifically that: -

(i) Circulating EPC numbers and function (assessed by flow cytometry and CFU-EPC assay) would vary during the menstrual cycle, healthy pregnancy and in pregnancies complicated by pre-eclampsia.

(ii) Circulating EPC numbers and function would correlate with serum hormone levels (oestradiol and progesterone) and measures of inflammation, IL-6, TNF-α, soluble ICAM-1 and VEGF.

The aim of this research was to make serial measurements of number and function of circulating EPCs together with circulating sex steroids and inflammatory mediators throughout the normal menstrual cycle, healthy pregnancy, and pregnancy affected by pre-eclampsia (Chapter 5).

1.9.3 ARTERIAL COMPLIANCE AND STIFFNESS

It was hypothesised that both central and systemic arterial stiffness will be altered in the fluctuating hormonal milieu of the menstrual cycle and pregnancy and the hypertensive state of pre-eclampsia (Chapter 6).

The aim of this research was to make serial measurements of reproductive hormones, central and systemic arterial stiffness throughout the normal menstrual cycle, healthy pregnancy, and pregnancy affected by pre-eclampsia.

CHAPTER 2

GENERAL METHODS

All materials, reagents and cell lines used are detailed in Appendix 1.
Recipes for reagents are provided in Appendix 2.

2.1 SUBJECT RECRUITMENT

2.1.1 ETHICAL APPROVAL AND CONSENT

Approval from the Lothian Local Research Ethics Committee (Reference numbers 04/S1103/40 and 05/S1104/48) was granted for all the experimental work described in this thesis. Two clinical studies were performed. The first (study 1) involved non-pregnant and healthy pregnant women, who underwent venous occlusion plethysmography (described below) and is detailed in chapter 3. The second (study 2) involved non-pregnant, healthy pregnant and pre-eclamptic women, who underwent venepuncture and arterial stiffness studies (described below) and is detailed in chapters 4,5 and 6. Women participated in either study 1 or study 2. Written information was provided and informed consent obtained from all study participants. All studies were undertaken in accordance with the Declaration of Helsinki.

2.1.2 NON-PREGNANT WOMEN

Healthy non-pregnant, nulligravida women with at least a two-month history of normal regular menstrual cycles were recruited from the University of Edinburgh staff and student populations. Exclusion criteria included the use of hormonal contraception, current or past heavy menstrual bleeding or dysmennorhoea, current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation. Non-pregnant women participated either in study 1(Reference number 04/S1103/40) or in study 2 (Reference number 05/S1104/48).

2.1.3 PREGNANT WOMEN

Healthy primigravida women were recruited from community antenatal clinics. Exclusion criteria included current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation. Pregnant women participated either in study 1(Reference number 04/S1103/40) or in study 2 (Reference number 05/S1104/48).

Primigravida women presenting with pre-eclampsia were recruited from the Obstetric unit, The Royal Infirmary of Edinburgh. Pre-eclampsia was defined by International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria [Brown *et al*, 2001] as a systolic blood pressure of 140 mmHg and a diastolic pressure (Korotkoff V) of 90 mmHg on at least two occasions after the 20th week of pregnancy in a previously normotensive woman and proteinuria (2+ on dipstick or an Albumin: Creatinine Ratio >30 mg/mmol). Exclusion criteria for women with pre-eclampsia included diabetes mellitus and multiple pregnancy. Women with pre-eclampsia were divided into pre-term and term groups on diagnosis being made before or after 34 weeks gestation respectively. This division was made due to its accepted use in the literature for early and late-onset pre-eclampsia [Egbor *et al*, 2006]. Women with pre-eclampsia participated in study 2 only (Reference number 05/S1104/48).

2.2 ARTERIAL STIFFNESS STUDIES

The following sensitive and reproducible methods [Wilkinson *et al*, 1998; Oliver and Webb, 2003] were used to provide measures of systemic and central arterial stiffness in accordance with the recommendations made by the Expert Consensus document on arterial stiffness [Laurent *et al*, 2006].

All studies were performed in a quiet, temperature controlled room (22-25°C) within the Wellcome Trust Clincal Research Facility. Subjects rested in the supine position or 30° left-lateral position (to avoid vena cava compression by the uterus) for non-pregnant and pregnant subjects, respectively. Blood pressure and heart rate were recorded in duplicate using an automated sphygmomanometer (Micro life 3BTO-A) validated for use in pregnancy and pre-eclampsia [Reinders *et al*, 2005], following a 30-minute rest period. During this rest period, venepuncture was performed for platelet and monocyte activation studies (chapter 4) and for endothelial progenitor cell studies (chapter 5).

2.2.1 PULSE WAVE ANALYSIS

Pulse wave analysis was performed using micromanometer applanation tonometry of the radial artery at the wrist and the SphygmoCor system, in accordance with the manufacturer's recommendations (www.atcormedical.com/technical notes.htm).

Pulse wave analysis derives an aortic pulse pressure waveform from the radial artery wave via a mathematical transfer function. The arterial pressure waveform is a

composite of the forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance. The augmentation index (AIx), which is the difference between the second and first systolic peaks, expressed as a percentage of the pulse pressure, is a measure of systemic arterial stiffness and wave reflection (Figure 7). Compliance is inversely proportional to AIx [Yasmin and Brown, 1999].

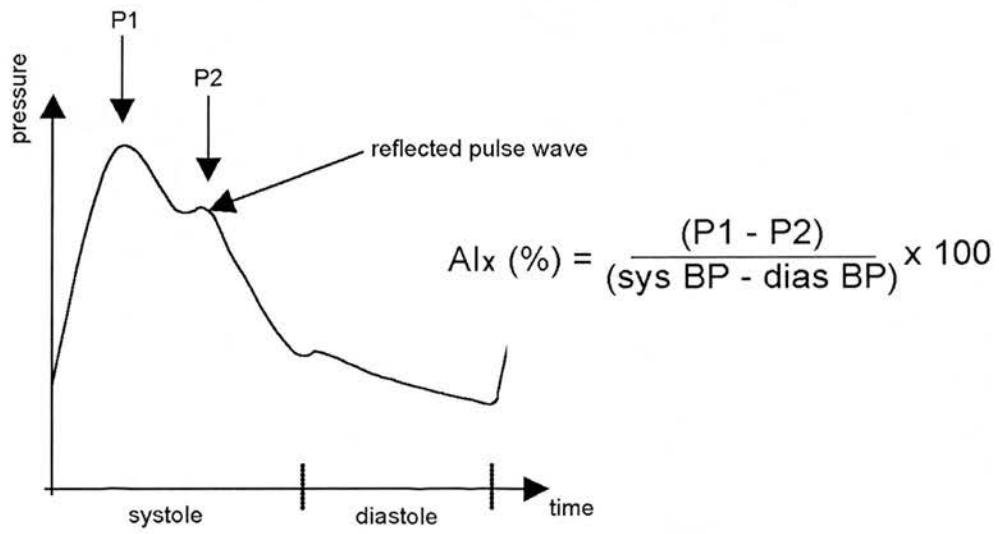

Figure 7. Average aortic pulse waveform from a young person. Augmentation index, expressed as a percentage, is the proportion of the pulse pressure, which is due to the reflected pulse wave.
AIx - augmentation index; sys BP - systolic blood pressure; dias BP - diastolic blood pressure.

At least two independent waveform analyses were obtained from each subject. Nursing staff, specifically trained in the technique from the Wellcome Trust Clinical Research Facility, performed all vascular measurements. These were only accepted on meeting SphygmoCor quality control criteria (average pulse height ≥100 units, pulse height variation ≤5%, diastolic variation ≤5%, quality index ≥80%).

2.2.2 PULSE WAVE VELOCITY

The same micromanometer and SphygmoCor system were used to assess carotid-femoral PWV, and carotid-radial PWV by applanation tonometry. The PWV was determined by sequential acquisition of pressure waveforms from the carotid, femoral and radial arteries. The timing of these waveforms was compared with that of the R wave on the simultaneously recorded electrocardiogram. The computer software measured the time delay automatically. The distance travelled by the pulse wave between the carotid artery and the femoral artery was measured in a straight line using a pair of compasses to reduce the influence of altered body contours due to pregnancy. The proximal distance was measured from the sternal notch to the sampling site on the carotid artery and the distal distance was measured from the sternal notch to the sampling site on the femoral artery (Figure 8). The carotid-to-femoral path length was estimated by subtracting the proximal from the distal distance.

Pulse wave velocity was then calculated as the ratio of the distance travelled by the pulse wave and the foot-to-foot time delay between the pulse waves, expressed in metres per second (m/s). For carotid-radial PWV the distal distance was measured from the sternal notch to the sampling point on the radial artery.

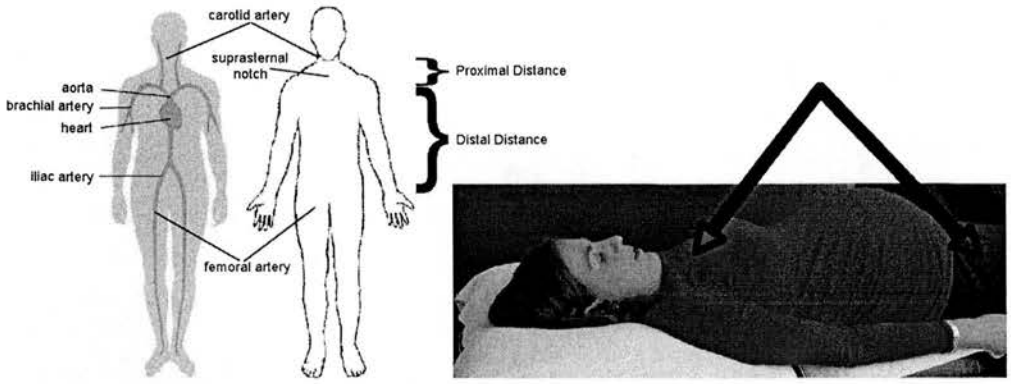

Figure 8. Distance travelled by the pulse wave between the carotid and femoral arteries. The proximal distance is measured between the suprasternal notch and the sampling point over the carotid artery. The distal distance is measured from the suprasternal notch to the sampling point over the femoral artery. A pair of compasses were designed and made to measure this in all subjects.

Arterial blood pressure varies with respiration so the average of ten successive measurements was used in the analyses to cover a complete respiratory cycle. For each subject, a total of two consecutive waveform recordings were obtained. The mean of these two readings were used in further analyses. Again, nursing staff specifically trained in the technique, performed all vascular measurements. Measurements were only accepted on meeting SphygmoCor quality control criteria (Standard deviation (metres per second (m/s)) ≤ 6% of the mean time).

2.3 VENOUS OCCLUSION PLETHYSMOGRAPHY

The basic methodology of venous occlusion plethysmography was first described in 1909 and, when combined with intra-arterial drug administration it is considered the gold standard technique for assessing vascular function in humans *in vivo* [Wilkinson and Webb, 2001b]. The technique is most often performed in the forearm, though it

can also be done in the lower limb. The underlying principle is as follows: brief interruption of venous drainage from the forearm, without affecting arterial inflow results in an increase in forearm volume. This increase is linear and proportional to arterial blood inflow, until venous pressure rises towards occluding pressure. At rest, approximately 70% of total forearm blood flow (FBF) is through skeletal muscle, most of the remainder is through the skin. Blood flow in the hand is different; therefore exclusion of the hand from the model is standard practice. If the hand is not excluded, the blood flow is nonlinear.

2.3.1 Method

Studies were carried out in a quiet temperature controlled room (22-25°C). The study was conducted in the supine position for non-pregnant women but pregnant women had a slight left-lateral tilt to minimise caval compression by the gravid uterus. Blood pressure and heart rate were recorded throughout the study using a semi-automated noninvasive oscillometric sphygmomanometer (OMRON 705 IT).

Both arms were positioned above the level of the right atrium to allow adequate venous drainage, with the elbows resting on foam pads and the hands supported on pillows. Inflatable cuffs were placed around both wrists and upper arms. The wrist cuffs were inflated to 200 mmHg (above systolic pressure) to exclude the hands from the circulation. The upper cuffs were inflated to 40 mmHg (above venous pressure, but below diastolic pressure). The upper cuffs were inflated for 7 seconds at a time and then deflated for 3 seconds to allow venous emptying. The wrist cuffs were

inflated for 3 minutes at a time and for at least 60 seconds before flow measurements were analysed, to allow FBF to stabilise. Mercury-in-silastic strain gauges were placed at the widest part of each forearm and connected to a plethysmograph. Change in forearm volume results in a corresponding change in circumference detected as an alteration in electrical resistance and thus potential difference. The gauge lengths were equal to the resting arm circumference (measured at the start of the study), so that changes in limb volume were directly proportional to the change in resistance. Venous occlusion plethysmography provides a measure of blood flow to that part of the forearm enclosed by the two cuffs, expressed as millilitre (mL) per 100 mL of forearm volume per minute. Within subject reproducibility for FBF, coefficient of variation is 10% [Wilkinson and Webb, 2001b].

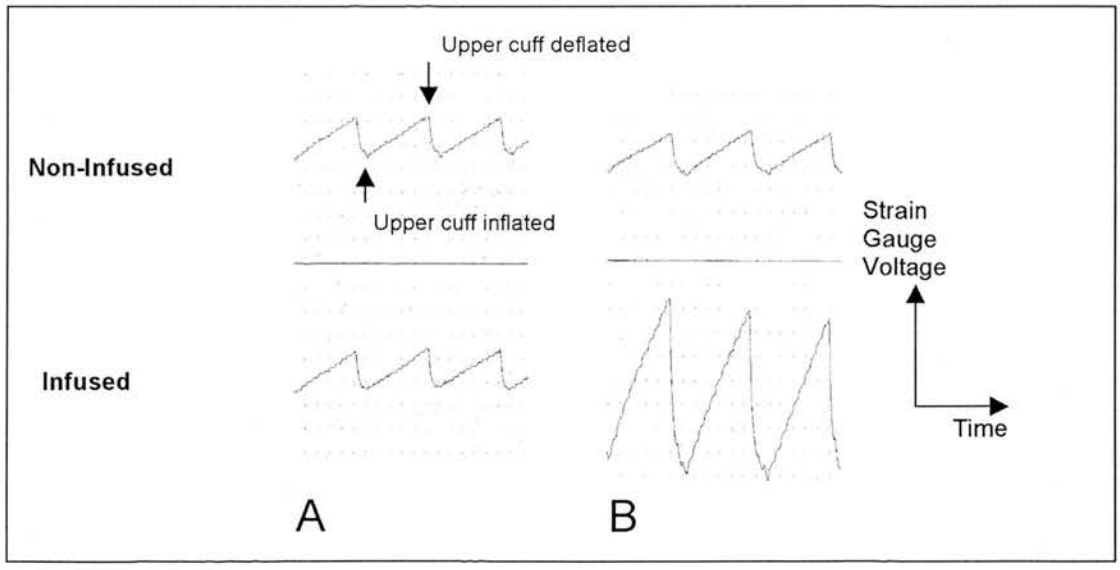

Figure 9. (2.3)The effect of an intra-arterial infusion of substance P on forearm blood flow. A) Intra-arterial saline infusion. B) Intra-arterial substance P infusion. Substance P produces a marked increase in blood flow in the infused arm, as illustrated by the increase in the slope of the tracing. Adapted from Wilkinson and Webb, 2001b [Wilkinson and Webb, 2001b].

At the start of the study, unilateral brachial artery cannulation with a 27-standard wire gauge steel needle was performed under controlled conditions [Wilkinson and Webb, 2001b], using local anaesthesia. Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms for collection of venous effluent (Figure 10). This allowed for the study of the local actions of drugs in the forearm vascular bed. Drugs were given at doses 10 to 100 times below those causing systemic effect, because FBF is approximately 100 times lower than cardiac output. The non-infused arm acts as a control to take into account any minor changes in both arms affecting blood flow such as emotional response, change in temperature.

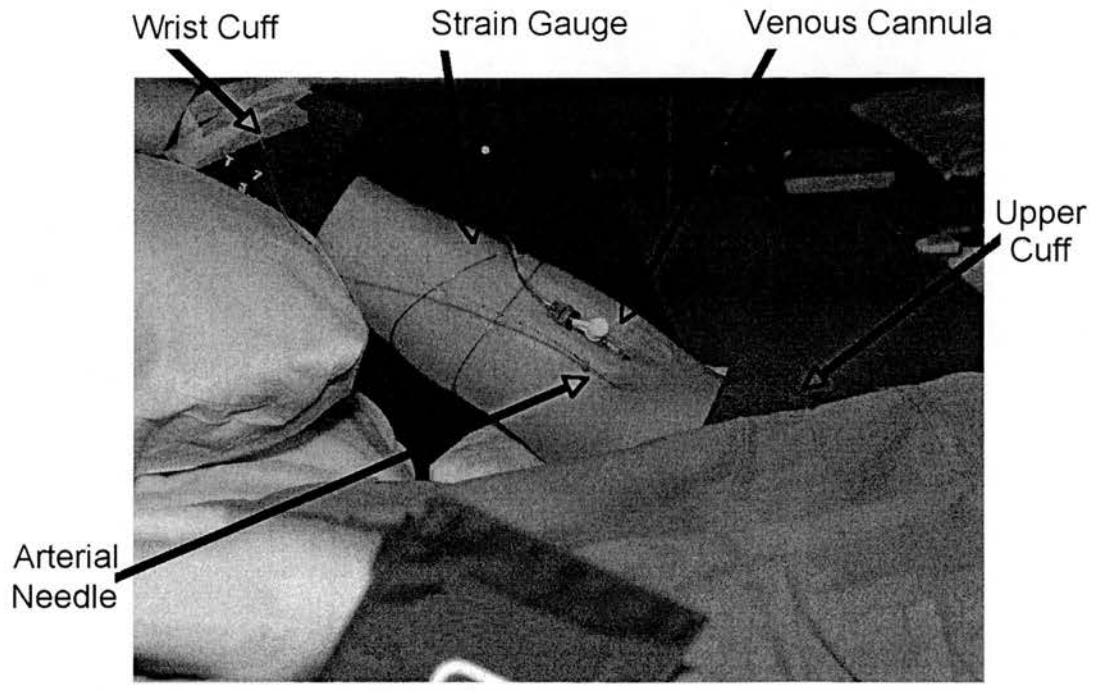

Figure 10. Photograph of subject demonstrating placement of cuffs, mercury-in-silastic strain gauges, arterial needle and venous cannulae.

2.3.2 DATA ANALYSIS

Plethysmographic data and net t-PA release were determined as described previously [Newby *et al*, 1997; Oliver *et al*, 2005]. Data were extracted from the Chart data files and FBFs were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 11.3). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the reflex vasoconstriction this causes. The last five linear flow recordings in each 3-minute measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit (Hct), and the infused forearm blood flow, (FBF)) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused ($[t\text{-}PA]_{Non\text{-}inf}$) arms:

$$\textbf{Estimated net t-PA release} = \textbf{FBF} \times (1\text{-}\textbf{Hct}) \times ([t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf})$$

The t-PA/PAI-1 ratio was calculated from the respective antigen concentrations.

2.4 SAMPLE COLLECTION

2.4.1 VENOUS BLOOD WITHIN FOREARM VENOUS PLETHYSMOGRAPHY STUDIES

Venous blood (10 mL) was drawn simultaneously from both arms at baseline and at defined points in the study protocol (Chapter 3) into acidified buffered citrate (Stabilyte tubes) and into sodium citrate (for t-PA and PAI-1 assays, respectively).

Samples were kept on ice before being centrifuged at 2000 g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay.

2.4.2 VENOUS BLOOD FROM ALL OTHER STUDIES

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle using minimal tourniquet pressure. Care was taken to ensure a smooth blood draw as previously described [Harding *et al*, 2007].

The first 3 mL of venous blood were discarded. Thereafter 3 mL of blood were collected in a tube containing the direct thrombin inhibitor D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75 μM, PPACK). This was chosen for its reliable anticoagulation without cation chelation (which reduces platelet-leukocyte interactions *in vitro*) or causing platelet activation [Harding *et al*, 2007]. Then 25 mL of blood were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) (1.6 mg/mL), and 10 mL in tubes containing sodium citrate. Finally 10 mL of blood were collected in serum gel tubes. Initial processing of samples occurred within 5 minutes of phlebotomy. Plasma was separated by centrifugation (2000 x g for 30 min) from blood anticoagulated with EDTA and sodium citrate. Serum was prepared from blood collected in the serum gel tubes. Plasma and serum samples were stored at -80°C until analysis. Five millilitres of blood anticoagulated with EDTA were sent to the hospital haematology laboratory for automated calculation of the full blood count differential.

2.5. FLOW CYTOMETRIC ANALYSIS

Flow cytometry is a technique that allows rapid measurements to be performed on single cells as they flow in a fluid stream past a sensing point. The important feature is that measurements are made on each cell in turn, not just as average values for the population. In the subsequent studies we have utilised flow cytometry to identify sub populations of cells in human venous blood and the subsequent cell surface expression of different molecules. We performed immunolabelling of cells in whole blood through incubation with combinations of fluorescent-conjugated antibodies. Thereafter samples were fixed (with, or without lysis of erythrocytes) until analysis. A laser flow cytometer was used. The laser light source illuminates cells within the sample of interest in addition to producing specific wavelength to excite the fluorescent dyes. Photon detectors collect the scattered and fluorescent light generated by the cells passing through an illuminating beam. These in turn convert photon pulses into electronic signals, which can be displayed graphically as histograms or dot plots. The following protocols (detailed in Appendix 3) have been developed and validated by our group and used in various study areas [Harding *et al*, 2004; Robinson *et al*, 2006; Harding *et al*, 2007; Tura *et al*, 2007].

2.5.1 PLATELET-MONOCYTE AGGREGATES

Platelet-monocyte aggregates are sensitive markers of platelet activation with important functional consequences [Michelson *et al*, 2001].

Immunolabelling was performed at exactly 5 minutes to minimise *ex vivo* platelet activation. All antibodies were used at 1:20 concentration in flow buffer (Appendix 2.) To quantify platelet-monocyte aggregates, 60 μL aliquots of whole blood, (anticoagulated with PPACK) were incubated with 60 μL of antibodies against CD14, CD42a and isotype matched controls for 20 minutes in darkness at room temperature. Thereafter samples were fixed and the red cells lysed by the addition of 500 μL of FACS-Lyse solution. Samples were stored at 4°C in the dark until analysis. Samples were analysed using a Becton Dickinson (BD) FACSCalibur Flow Cytometer equipped with a 488 nm wavelength laser within 12 hours of labelling. Monocytes were identified by their forward and side scatter properties and CD14 expression. Two thousand, five hundred cells were collected. Platelet-monocyte aggregates were defined as monocytes positive for the platelet marker CD42a. The mean coefficient of variation for the percentage of platelet-monocyte aggregates was 7.8%.

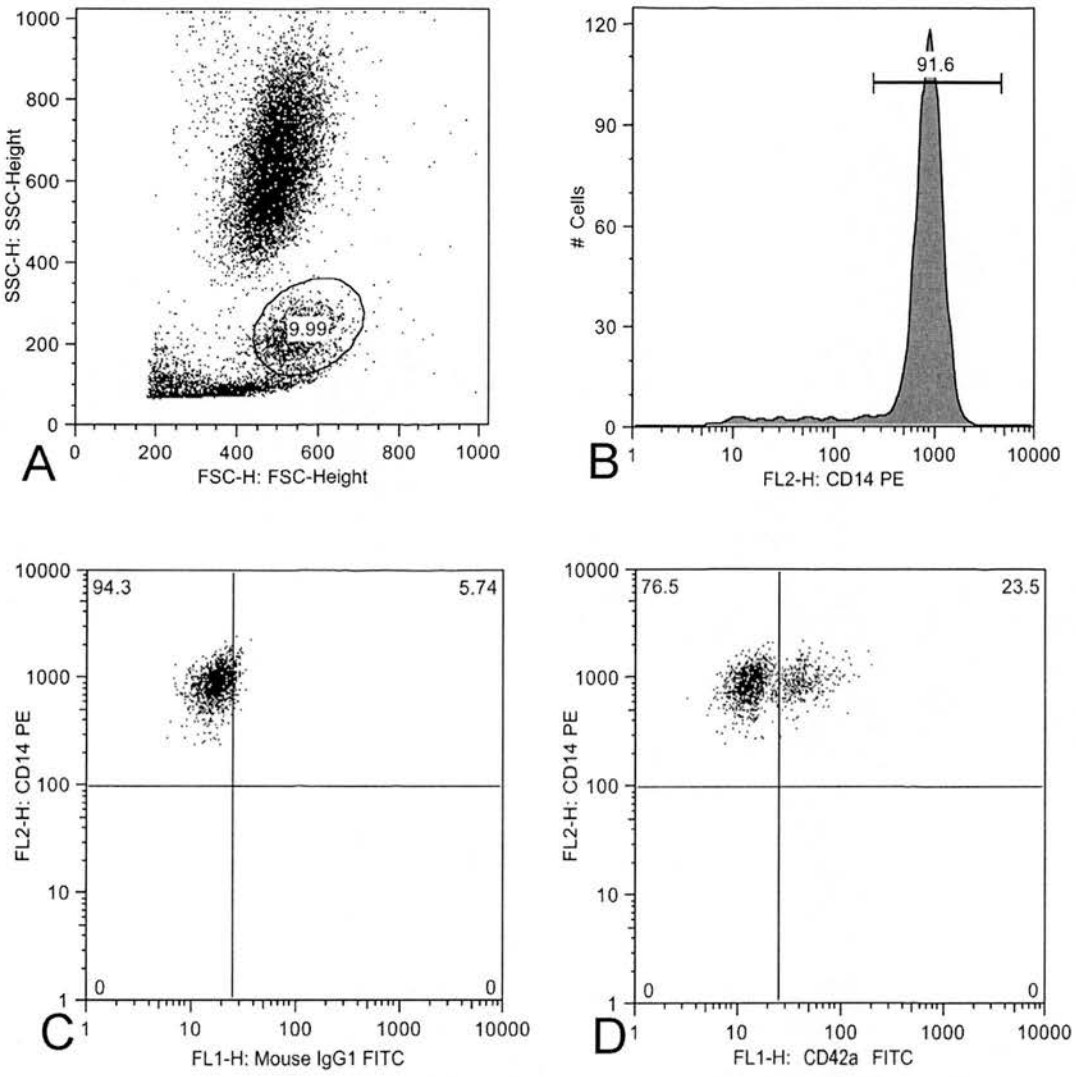

Figure 11. Representative flow cytometric analysis of platelet-monocyte aggregates from non-pregnant subject. Monocytes were labelled with CD (cluster of differentiation)14-PE (phycoerythrin) and platelets with CD42a-FITC (fluorescein isothiocyanate). (a) Monocytes were identified by their characteristic forward- and side-scatter properties. Monocyte population in 'gated' oval. (b) A histogram of gated events was created, with a second gate placed over the CD14-bright monocytes. A total of 2,500 CD14-bright monocytes were collected for analysis. (c) A quadrant plot of CD14-bright monocytes against an appropriate Ig (immunoglobulin)G1 isotype control was used to set a marker to allow for non-specific binding and autofluorescence. (d) A second quadrant plot of CD14-bright monocytes against CD42a was then used to determine the percentage of monocytes positive for CD42a.
SSC - side-scatter; FSC - forward-scatter; FL - fluorescence parameter.

2.5.2 PLATELET SURFACE P-SELECTIN EXPRESSION

Platelets rapidly express P-selectin on their cell surface when activated [van Gils *et al*, 2009].

Immunolabelling was performed at exactly 5 minutes to minimise *ex vivo* platelet activation. All antibodies were used at 1:20 concentration in flow buffer (Appendix 2). To quantify platelet surface P-selectin, 5 µL aliquots of whole blood (anticoagulated with PPACK) were incubated with 45 µL of antibody against CD42a, CD62P and isotype matched controls for 20 minutes in darkness at room temperature before samples were fixed by the addition of 1450 µL of 1% paraformaldehyde (Appendix 2). Light scatter and CD42a expression identified platelets, and 7500 cells collected.

2.5.3 MONOCYTE ACTIVATION

CD11b and CD40 expression are markers of activated monocytes [van Gils *et al*, 2009].

Immunolabelling was performed at exactly 5 minutes to minimise *ex vivo* platelet activation. All antibodies were used at 1:20 concentration in flow buffer (Appendix 2). Monocyte CD40 and CD11b expression were evaluated by incubation of 60 µL of whole blood (anticoagulated with PPACK) with 60 µL of antibody against CD14, CD40 and CD11b for 20 minutes in darkness at room temperature, before 500 µL of FACS-Lyse was added. Light scatter and CD14 expression identified monocytes. 2500 cells were collected.

Results are expressed as percentage of positive cells. Monocyte CD11b expression was quantified using mean fluorescent intensity (MFI) due to its constitutive presence. Analyses were performed using Flowjo software (Tree Star, Inc. Oregon, USA).

2.5.4 ENDOTHELIAL PROGENITOR CELLS

To quantify endothelial progenitor cells, defined as cells co-expressing CD34, CD133 and VEGF receptor 2 (KDR); three-colour flow cytometry was used. In early studies, isotype-matched negative control antibodies were compared with unstained samples. Since these did not differ, unstained samples were used to establish positive stain boundaries for CD34 and CD133 expression [Tura *et al*, 2007]. For KDR expression, the appropriate isotype control immunoglobulin (Ig)G1 was used.

Two hundred millilitre aliquots of whole blood, (anticoagulated with EDTA) were incubated with 5 μL aliquots of mouse anti-human CD34-fluorescein isothiocyanate (FITC), mouse anti-human CD133-phycoerythrin (PE), mouse monoclonal anti-human VEGF receptor 2 (KDR)-allophycocyanin (APC) and mouse IgG1 isotype control-APC for 20 minutes in the dark at room temperature. Samples were stained with none, single, double and triple antibodies over the sequence, none, CD34, CD133, KDR or IgG1 isotype control in a 5 tube panel. Thereafter, samples were fixed and erythrocytes lysed by the addition of 200 μL of UTI-Lyse reagent A. After 10 minutes, 2 mL of UTI-Lyse reagent B were added. Ten minutes later, cells were washed and separated by centrifugation at 1500 x g for 5 minutes at room

temperature. The supernatant was decanted and 1 mL of phosphate buffered saline (PBS) without cations added. The washing process was repeated as before and finally cells were resuspended by adding 500 μL of PBS to each tube.

Samples were stored in darkness at room temperature until flow cytometric analysis within 12 hours of labelling. Analysis was performed on a BD FACSCalibur Flow Cytometer equipped with 488 nm and 635 nm wavelength lasers connected to an Apple MacIntosh computer equipped with CellQuest software. Consistent with protocols for the identification of rare cells [Donnenberg and Donnenberg, 2007], distilled water was run between each sample and a low flow rate (12 μL/min) was used to minimise coincident events. The flow cytometer was calibrated and compensated at frequent intervals with calibration beads and software appropriate for the flurocromes employed, in accordance with the manufacturer's recommendations.

The identical stored collection settings were used for all samples. For each sample, 80,000 events were acquired in the lymphocyte region (as determined by characteristic forward- and side-scatter profile), but all events were saved for analysis.

Data was saved as listmode files and transferred to another Apple MacIntosh computer for detailed analysis, using Flowjo software. Boolean gating was employed (Figure 12). Endothelial progenitor cells were identified by the co-expression of CD34, CD133, and KDR antigens and quantified as a percentage of total leucocytes.

Absolute numbers were determined through multiplication using the total white count and triple positive leucocytes expressed as number of cells per litre of blood.

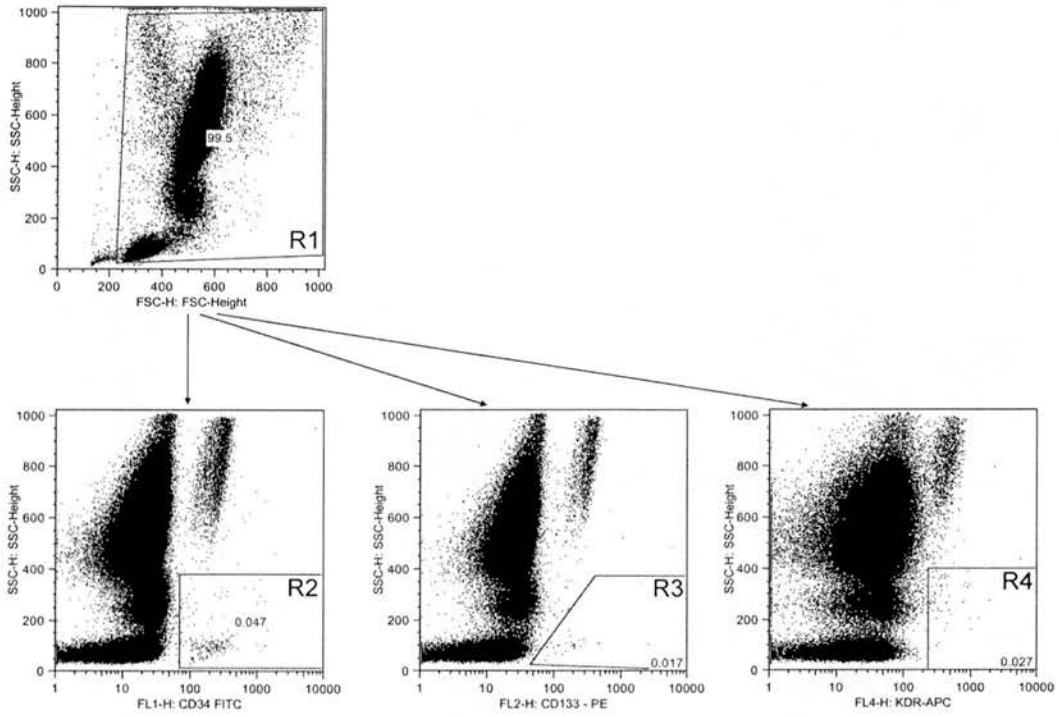

Figure 12. Flow cytometric analysis of endothelial progenitor cells in whole blood. Cells were labelled with CD (cluster of differentiation)34-FITC (fluorescein isothiocyanate), CD133-PE (phycoerythrin), KDR-APC (kinase insert domain receptor-allophycocyanin) or Ig (immunoglobulin)G1-APC. Representative forward- and side-scatter properties (cell size and granularity) of leucocytes in whole blood. Debris was excluded by primary gate R1. Events within R1, CD34 positive events within gate R2. CD133 positive events within gate R3 and KDR positive events within R4. Boolean gating used to identify cells present in all 3 gates, R2, R3 and R4 ('triple-positive' cells).
SSC - side-scatter; FSC - forward-scatter; FL - fluorescence parameter.

2.6 *IN VITRO* CULTURE

All cells were maintained at 37°C, 5% CO2 with 95% humidity.

2.6.1 CFU-EPC ASSAY

Based on the assay described by Hill *et al* [Hill *et al*, 2003], functional EPCs were quantified using the EndoCult™ CFU-EPC assay and commercial kit reagents according to the manufacturer's recommendations.

Venous blood samples (10 mL anticoagulated with EDTA) were diluted 1:1 with sterile PBS without cations and layered onto 15 mL of Histopaque. Samples were then spun at 1050 x g for 20 minutes at room temperature and the intermediate opaque mononuclear layer collected. The peripheral blood mononuclear cells (PBMCs) were washed twice with PBS, once with EndoCult™ complete culture medium (ECCM, Appendix 2) and 5×10^6 cells resuspended in 2 mL of ECCM. Cells were then plated at 2 mL/well in a 6-well fibronectin-coated plate and incubated at 37°C, 5% CO2 with 95% humidity for 2 days. The non-adherent cells were then harvested, and transferred in fresh ECCM to a 24-well fibronectin-coated plate at 1×10^6 cells/mL/well for a further 3 days. Colonies (CFU-EPCs), were defined using the published method as a central core of "round" cells surrounded by elongated "sprouting" cells at the periphery (Figure 13). Colonies were counted in a minimum of 2-wells per sample by observers unaware of the subjects' clinical profiles and the results expressed as mean numbers of CFU-EPCs per 1×10^6 cells plated.

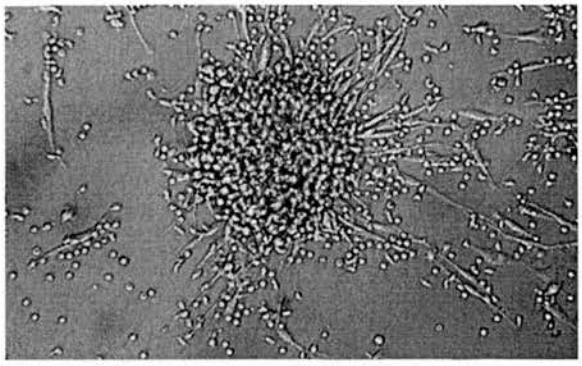

Figure 13. A Typical colony forming unit with a characteristic core of round cells and sprouting spindle cells at the periphery (x100 magnification).

2.6.2 HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS)

Dr C. Shaw, Department of Cardiovascular Research, The University of Edinburgh kindly provided these. Human umbilical vein endothelial cells (HUVECs) were cultured onto 4-well plain glass chamber slides at a density of $7x10^5$ cells in endothelium growth medium 2 before being fixed in methanol and stained as below.

2.6.3 HT-29, HUMAN COLON ADENOCARCINOMA CELL LINE

Dr S Bader, Edinburgh Cancer Research Centre, The University of Edinburgh kindly donated these cells. Cells were cultured in Rosewell Park Memorial Institute (RPMI) medium supplemented with 5% foetal calf serum until 80% confluence was achieved before being fixed and stained as below.

2.7 IMMUNOFLUORESCENCE

2.7.1 METHOD

To confirm endothelial cell lineage, colonies were stained directly using acetylated low density lipoprotein (LDL) and co-stained with lectin (UEA-1) as previously described [Kalka *et al*, 2000]. Cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylcarbocyanine-labelled acetylated LDL (Dil-AcLDL); 1:100 in ECCM for 4 hours, washed in PBS and fixed with ice-cold methanol for 10 minutes. Colonies were stained for 1 hour with FITC-labelled lectin from UEA-1 (1:100 in PBS), counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and mounted with Permafluor.

For CD105 (endoglin) and CD146 staining, fixed colonies were permeabilised and blocked in a solution of 10% goat serum, 1% bovine serum albumin and 0.02% IGEPAL/NP40. Next, colonies were incubated with primary murine monoclonal antibodies against human CD105 (1:500) or CD146 (1:200) for 2 hours at room temperature. A secondary polyclonal goat anti-mouse biotinylated antibody was added for 30 minutes followed by streptavidin-Alexa Fluor 546 or streptavidin-Alexa Fluor 488 for 1 hour in the dark. Nuclei were counterstained and colonies mounted as above. Between each step the slides were washed with PBS for 5 minutes. Human umbilical vein endothelial cells were used as positive controls for all of the endothelial markers. Staining was performed without the primary antibody as a negative control for each antibody. In addition the HT-29 human colon cell-line was used as a negative control for the endothelial markers. Phase-contrast images of the

same CFU-EPCs were used to confirm colony morphology. All images are representative of at least three different subjects (Figure 14).

2.7.2 CONFOCAL MICROSCOPY AND IMAGE ANALYSIS

A Zeiss 'LSM 510 Meta' confocal microscope, with x40 oil immersion objective was used to visualise the stained cells. Image capture was performed with LSM 510 meta-analysis computer software. Detection gain and offset setting were established on the positive controls and the same levels used on the CFU-EPCs and respective negative controls. Images for all antibodies were captured at single slices through the colonies. Z-stack images were also captured providing a 3D image of the colony through reconstitution of serial image captured at preset intervals.

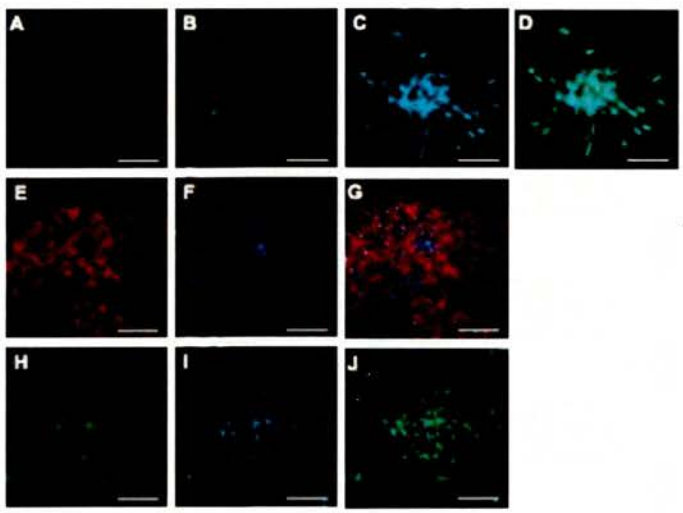

Figure 14. Characterisation of CFU-EPCs (colony forming unit-endothelial progenitor cells). Live CFU-EPCs exposed to Dil-AcLDL (1,1'-dioctadecyl-3,3,3',3'-tetramethylcarbocyanine-labelled acetylated low-density lipoprotein) (A), stained with FITC (fluorescein isothiocyanate)-lectin (Ulex europaeus agglutinin-1) (B), nuclear counter stain DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) (C) and merged image (D). Fixed CFU-EPCs immunostained for the endothelial cell marker CD (cluster of differentiation)105 (E), nuclear counterstain (F) merged image (G) and for CD146 (H), nuclear counterstain (I) and merged (J). (x 40 magnification)

2.8 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs can quantify the amount of a given protein in a solution, through comparison with a standard curve created from samples containing known concentrations. Most of the assays performed in this project were sandwich ELISAs.

A sandwich ELISA uses plates coated with an antibody raised against one epitope of the substance to be measured. The solution of interest, or the standard, is then added, and the immobilised antibody binds the substance. After washing away unbound substances, an enzyme-linked antibody specific for the substance to be measured is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, during which amplifier solutions are sometimes added, colour develops in proportion to the amount of substance bound in the initial step. The reaction is stopped with sulphuric acid solution and the absorbance measured by spectrophotometry.

Standard curves were constructed. Assays were validated by performing serial dilutions of a sample and confirming that when the absorbance was plotted a linear response was obtained which was parallel to the standard curve. Assay precisions were calculated using the formula "standard deviation/mean x 100" to give the coefficient of variation (%CV).

2.8.1 SOLUBLE P-SELECTIN ELISA

A soluble P-selectin assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Samples, controls and standards (100 µL) were diluted in dilutant provided and applied to each well. Diluted conjugate (sheep polyclonal antibody to recombinant human soluble P-selectin conjugated to horseradish peroxidase) (100 µL) was added to each well and incubated at room temperature for 1 hour. Plates were washed three times with wash buffer. Substrate (tetramethylbenzidine) was applied to each well (100 µL) and incubated at room temperature for 15 minutes. One hundred microlitres of stop solution was then added to each well and the plate read on a microplate reader (Thermomax, Molecular Devices, CA. USA) within 30 minutes. The intra-assay CV was 4.9%; the inter-assay CV was 7.9% and the detection limit of the assay was 0.5 ng/mL.

2.8.2 CD40 LIGAND ELISA

A CD40 ligand assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Samples, controls and standards (200 µL) were diluted in dilutant provided and applied to each well. Diluted conjugate (polyclonal antibody to recombinant human CD40L conjugated to horseradish peroxidase) (200 µL) was added to each well and incubated at room temperature for 2 hours. Plates were washed four times with wash buffer. Substrate (200 µL) was applied to each well and incubated at room temperature for 30 minutes in the dark. Fifty microlitres of stop solution was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 4.5%;

the inter-assay CV was 6.0% and the detection limit of the assay was 4.2 pg/mL.

2.8.3 IL-6 ELISA

An IL-6 assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Samples, controls and standards (200 μL) were diluted in dilutant provided and applied to each well and incubated at room temperature for 2 hours. Plates were washed 6 times with wash buffer. Diluted conjugate (polyclonal antibody to IL-6 conjugated to alkaline phosphatase) (200 μL) was added to each well and incubated at room temperature for 2 hours. Plates were washed a further 6 times with wash buffer. Substrate (lyophilized NADPH) (50 μL) was applied to each well and incubated at room temperature for 1 hour. Fifty microlitres of amplifier solution was applied to each well and incubated at room temperature for 30 minutes. Fifty microlitres of stop solution (sulfuric acid) was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 6.9%; the inter-assay CV was 6.5% and the detection limit of the assay was 0.039 pg/mL.

2.8.4 TNF-α ELISA

A tumour necrosis factor-α assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Samples, controls and standards (250 μL) were diluted in dilutant provided and applied to each well and incubated at room temperature for 3 hours. Diluted conjugate (polyclonal antibody to TNF-α conjugated to alkaline phosphatase) (200 μL) was added to each

well and incubated at room temperature for 2 hours. Plates were washed six times with wash buffer. Substrate (lyophilized NADPH) (50 μL) was applied to each well and incubated at room temperature for 1 hour. Fifty microlitres of amplifier solution was applied to each well and incubated at room temperature for 30 minutes. Fifty microlitres of stop solution (sulfuric acid) was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 3.1%; the inter-assay CV was 7.3% and the detection limit of the assay was 0.1 pg/mL.

2.8.5 SOLUBLE ICAM-1 ELISA

A soluble ICAM-1 assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Diluted conjugate (antibody to soluble ICAM-1 conjugated to horseradish peroxidase) (100 μL) was added to each well. Samples, controls and standards (100 μL) were diluted in dilutant provided and applied to each well and incubated at room temperature for 1.5 hours. Plates were washed 6 times with wash buffer. Substrate (100 μL) was applied to each well and incubated at room temperature for 30 minutes. One hundred microlitres of stop solution was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 3.3%; the inter-assay CV was 6.0% and the detection limit of the assay was 0.35 ng/mL.

2.8.6 VEGF-ELISA

A vascular endothelial growth factor assay kit was used, containing a single plate pre-coated with capture antibody, and all required reagents and buffers (Appendix 1). Samples, controls and standards (200 μL) were diluted in dilutant provided and

applied to each well and incubated at room temperature for 2 hours. Plates were washed three times with wash buffer. Diluted conjugate (antibody to VEGF conjugated to horseradish peroxidase) (200 µL) was added to each well and incubated at room temperature for 2 hours. Plates were washed three times with wash buffer. Substrate (200 µL) was applied to each well and incubated at room temperature for 25 minutes. Fifty microlitres of stop solution was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 6.5% and the detection limit of the assay was 9.0 pg/mL

2.8.7 T-PA ANTIGEN AND ACTIVITY ELISA

Plasma t-PA antigen and activity were determined using a commercially available combined assay kit, containing plates pre-coated with capture antibody that does not interfere with t-PA functional activity, and all required reagents and buffers (Appendix 1). In the first step of this assay, t-PA and t-PA-PAI-1 complexes are bound to the plate and active non-complexed t-PA is thereafter quantified using a solution of Glu-plasminogen, fibrinogen fragments and a synthetic substrate, which reacts with plasmin formed in the reaction to yield a coloured reaction product. The second part of this assay is used to determine the total amount of t-PA antigen bound to the plate through the use of a horseradish peroxidase-labelled monoclonal antibody.

Samples, controls and standards (100 µL) were diluted in dilutant provided and applied to each well and incubated at 4°C overnight. Plates were washed three times with wash buffer. Plasminogen activator detection mixture (200 µL) was added to

each well and incubated at 37°C for 1 hour. The plate was read on a microplate reader. Thereafter the plate was washed three times with wash buffer. Diluted conjugate (antibody to t-PA conjugated to horseradish peroxidase) (100 µL) was added to each well and incubated at 37°C for 1 hour. Plates were washed three times with wash buffer. Substrate (100 µL) was applied to each well and incubated at room temperature for 10 minutes. One hundred microlitres of stop solution was then added to each well and the plate read on a microplate reader within 30 minutes. The inter- and intra-assay CVs were 9.8% and 5% respectively. The detection limit of the assay was 0.05 units/mL for t-PA activity and 0.1 ng/mL for t-PA antigen.

2.8.8 PAI-1 ANTIGEN ELISA

Plasminogen activator inhibitor type 1 antigen was determined using a commercially available combined assay kit, containing plates pre-coated with capture antibody (monoclonal anti-PAI-1), and all required reagents and buffers (Appendix 1). Samples, controls and standards (200 µL) were diluted in dilutant provided and applied to each well and incubated at 37°C for 1 hour. Plates were washed four times with wash buffer. Diluted conjugate (antibody to PAI-1 conjugated to horseradish peroxidase) (200 µL) was added to each well and incubated at 37°C for 1 hour. Plates were washed four times with wash buffer. Substrate (200 µL) was applied to each well and incubated at room temperature for 30 minutes. Fifty microlitres of stop solution was then added to each well and the plate read on a microplate reader within 15 minutes. The intra-assay CV was 5.0%; the inter-assay CV was 6.5% and the detection limit of the assay was 0.5 ng/mL.

2.8.9 PAI-1 ACTIVITY ELISA

Plasminogen activator inhibitor type 1 activity was determined using a commercially available assay kit, containing plates pre-coated with capture antibody (recombinant t-PA), and all required reagents and buffers (Appendix 1). Samples, controls and standards (200 μL) were diluted in dilutant provided and applied to each well and incubated at room temperature for 1 hour. Plates were washed five times with wash buffer. Diluted conjugate (antibody to PAI-1 conjugated to horseradish peroxidase) (200 μL) was added to each well and incubated at room temperature for 1 hour. Plates were washed five times with wash buffer. Substrate (200 μL) was applied to each well and incubated at room temperature for 5 minutes. Fifty microlitres of stop solution was then added to each well and the plate read on a microplate reader within 30 minutes. The intra-assay CV was 5.5%; the inter-assay CV was 7.5% and the detection limit of the assay was ≤0.1ng/mL.

2.9 AUTOMATED IMMUNOASSAYS

2.9.1 OESTRADIOL

Oestradiol was measured in serum using the automated Abbott Architect Integrated system within the Clinical Biochemistry Department, The Royal Infirmary of Edinburgh. A chemiluminescent microparticle immunoassay was performed on samples. Serum samples from pregnant subjects were manually diluted prior to placement in the machine. The intra-assay CV was ≤7%; the inter-assay CV was ≤6 % and the detection limit of the assay was 10 pg/mL.

2.9.2 PROGESTERONE

Progesterone was measured in serum using the automated ADVIA Centaur® Immunoassay system within the Clinical Biochemistry Department, The Royal Infirmary of Edinburgh. A chemiluminescent microparticle immunoassay was performed on samples. Serum samples from pregnant subjects were manually diluted prior to placement in the machine. The intra-assay CV was ≤7 %; the inter-assay CV was ≤5 % and the detection limit of the assay was 0.67 nmol/L.

2.9.3 LUTENISING HORMONE

Lutenising Hormone was measured in serum using the automated Abbott Architect Integrated system within the Clinical Biochemistry Department, The Royal Infirmary of Edinburgh. A chemiluminescent microparticle assay was performed on samples. The intra-assay CV was ≤ 3%; the inter-assay CV was ≤ 3% and the detection limit of the assay was 0.07 mIU/mL.

2.9.4 FOLLICLE-STIMULATING HORMONE (FSH)

Follicle-stimulating hormone was measured in serum using the automated Abbott Architect Integrated system within the Clinical Biochemistry Department, The Royal Infirmary of Edinburgh. A chemiluminescent microparticle assay was performed on samples. The intra-assay CV was ≤ 3%; the inter-assay CV was ≤ 2.7% and the detection limit of the assay was 0.05 mIU/mL.

2.10 STATISTICAL ANALYSIS

Continuous variables are reported as mean±standard error of the mean (SEM). Continuous variables were analysed using the Kolmogorov-Smirnov test for normality. Statistical analyses were performed using one and two-way analysis of variance (ANOVA) with repeated measures and Bonferroni's post-tests for multiple comparisons or two-tailed Student's *t*-test where appropriate. Friedman analyses and Mann-Whitney tests were used for non-parametric data. Correlation coefficients were calculated using Pearson or Spearman analyses for parametric and non-parametric data respectively. All calculations were performed using GraphPad Prism. Statistical significance was taken at 5%.

Graphs of parametric data represent mean±SEM. Graphs of non-parametric data represent median±interquartile range.

CHAPTER 3

ACUTE ENDOTHELIAL TISSUE PLASMINOGEN ACTIVATOR RELEASE IN PREGNANCY

Robb AO, Mills NL, Din JN, Cameron S, Ludlam CA, Newby DE, Denison FC.
Acute endothelial tissue plasminogen activator release in pregnancy.
J Thromb Haemost 2009;**7** (1):138-142.

3.1 INTRODUCTION

Pregnancy is recognised as a hypercoagulable state that protects women from potentially catastrophic haemorrhage during placentation and the post-partum period [Hellgren, 2003]. However, this predisposes women to thrombosis and thromboembolism [Lewis 2005] with many complications of pregnancy either being caused or exacerbated by the hypercoagulable state [Gilabert *et al*, 1995].

The pro-thrombotic state of pregnancy has been attributed to marked alterations in the coagulation and fibrinolytic system, including increased plasma concentrations of t-PA and its principal inhibitor, PAI-1 [Hellgren, 2003]. The relative balance between plasma t-PA and PAI-1, and the acute release of t-PA from the endothelium determines the efficacy of endogenous fibrinolysis [Oliver *et al*, 2005]. Basal plasma t-PA concentrations do not reflect the local capacity for acute endothelial t-PA release [Hrafnkelsdottir *et al*, 2004]. This underscores the importance of assessing acute local endothelial t-PA release, which is of greater pathophysiological relevance.

No studies have previously assessed the acute endothelial release of t-PA during pregnancy. The aim of work presented in this Chapter was, therefore, to assess whether impairment of acute endogenous fibrinolytic capacity contributes to the pro-thrombotic consequences of pregnancy.

3.2 METHODS

All materials and reagents used are detailed in Appendix 1.

3.2.1 SUBJECT RECRUITMENT

Women were identified, recruited and consented to this study (study 1, Reference number 04/S1103/40), as described in section 2.1.

Non-pregnant women

Healthy pre-menopausal nulliparous women (n=20) with at least a 2-month history of normal regular menstrual cycles were recruited to the study. Exclusion criteria included the use of hormonal contraception, current or past heavy menstrual bleeding or dysmennorhoea, current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation as detailed in section 2.1.2.

Pregnant women

Healthy primigravida women with an uncomplicated pregnancy (n=10) were recruited in the third trimester of pregnancy. Exclusion criteria included current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation as detailed in section 2.1.3.

3.2.2 VISIT SCHEDULE

Non-pregnant women

Women attended for a single visit in the mid-follicular phase (day 9.8±0.3) of their menstrual cycle.

Pregnant women

Women attended for a single visit during the third trimester of pregnancy (week 36±1).

3.2.3 STUDY PROTOCOL

Women abstained from alcohol, caffeine and tobacco for 24 hours and fasted for 4 hours prior to attendance. Prior to commencement of the study, height and weight were measured to allow calculation of body mass index (BMI).

Studies were carried out in a quiet temperature controlled room (22-25°C). The study was conducted in the supine position for non-pregnant women but pregnant women had a slight left-lateral tilt to minimise caval compression by the gravid uterus. Blood pressure and heart rate were recorded throughout the study using a semi-automated noninvasive oscillometric sphygmomanometer.

Venous occlusion plethysmography

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described in section 2.3.1, in conjunction with intra-arterial drug administration, detailed below.

Intra-arterial drug administration

All women underwent unilateral brachial artery cannulation with a 27-standard wire gauge steel needle as described in section 2.3.1. All studies commenced with a 20-minute saline infusion. Following this the following drugs (dilutions detailed in Appendix 2) were infused in a randomised order:

Bradykinin (endothelium-dependent vasodilator that stimulates the release of t-PA) was infused at 100, 300 and 1000 pmol/min and sodium nitroprusside (endothelium-independent vasodilator that does not stimulate release of t-PA) was infused at 2, 4 and 8 μg/min. The two vasodilators were separated by a 20-minute saline infusion and given in a randomised order (Figure 15).

Assessment of acute t-PA release

Venous cannulae were inserted into large subcutaneous veins of the antecubital fossa in both arms. Blood (10 mL) was drawn simultaneously from both arms at baseline and after each dose of bradykinin into acidified buffered citrate (for t-PA assays) and into citrate (for PAI-1 assays) (Figure 15). Samples were collected, processed and stored as described in section 2.4.1.

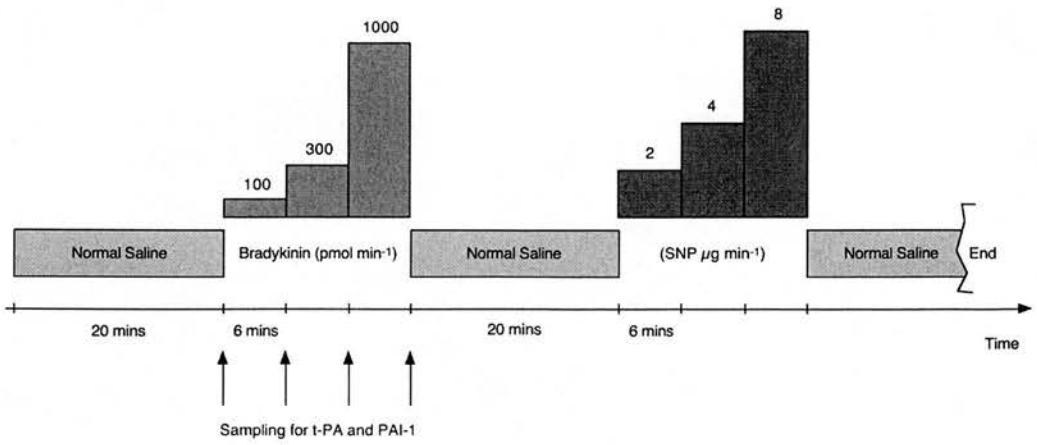

Figure 15. Schematic diagram demonstrating the intra-arterial drug administration protocol and time points for venous blood sampling for t-PA and PAI-1 assays. Bradykinin and sodium nitroprusside were given in a randomised order within each study.
SNP - sodium nitroprusside; t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type 1.

3.2.4 Measurement of t-PA and PAI-1 antigen and activity concentrations

Plasma t-PA antigen and activity and PAI-1 antigen and activity concentrations were measured in plasma using commercially available enzyme-linked immunosorbent assays as detailed in sections 2.8.7-9.

3.2.5 Measurement of circulating hormones

At the commencement of the study, venous blood (10 mL) was collected in a serum gel tube, centrifuged and serum samples were stored at -80°C until analysis. Serum concentrations of oestradiol and progesterone were measured in all women. Luteinizing hormone and FSH were also measured in the healthy non-pregnant women. All hormones were measured by automated chemiluminescent microparticle assays as described in section 2.9.1-4.

3.2.6 Data analysis

Plethysmographic data and net t-PA release were determined as described in section 2.3.2.

3.2.7 Statistical analysis

Continuous variables are reported as mean±SEM using ANOVA with repeated measures and two-tailed Student's *t*-test where appropriate.

All calculations were performed using GraphPad Prism, (GraphPad Software, USA). Statistical significance was taken at 5%.

3.3 RESULTS

3.3.1 BASELINE CHARACTERISTICS

Due to technical problems, one pregnant woman and three non-pregnant women were unable to complete the complex vascular studies. Pregnant (n=9) and non-pregnant (n=17) women were well-matched for age (26±3 *versus* 27±1 years, p=0.67) and BMI (24±1 kg m^{-2} *versus* 24±1 kg m^{-2}, p=0.77). Serum hormonal assays confirmed that the non-pregnant women were studied in the mid-follicular phase of their menstrual cycle. All pregnant women had uncomplicated pregnancies and delivered at term (mean gestational age at delivery of 40±1 weeks) with average birth weights (3,144±186 g). Three pregnant and three non-pregnant women were unable to complete the fibrinolytic studies due to technical problems with simultaneous venous access leaving a final study group of six pregnant and fourteen non-pregnant women for fibrinolytic analysis. These women were well-matched for age (26±4 *versus* 27±2 years, p=0.94), BMI (24±1 kg m^{-2} *versus* 24±1 kg m^{-2}, p=0.85) and current smoking status (2/6 *versus* 3/14, p=0.6).

3.3.2 VASOMOTOR FUNCTION

There were no differences in resting systolic (122±3 *versus* 116±2 mmHg, p=0.14) or diastolic blood pressure (76±3 *versus* 73±2 mmHg, p=0.39) between pregnant and non-pregnant women. Similarly, there was no difference in basal infused (3.1±0.5 *versus* 2.1±0.2 mL^{-1} 100 mL^{-1} min^{-1}, p=0.07) or non-infused (2.4±0.4 *versus* 1.8±0.2 mL^{-1} 100 mL^{-1} min^{-1}, p=0.11) forearm blood flow between pregnant and non-

pregnant women. However, consistent with a hyperdynamic circulation, pregnant women had increased resting heart rates compared to non-pregnant women (80±3 *versus* 67±2 bpm, p=0.002).

There was a dose-dependent increase in forearm blood flow with bradykinin ($p<0.0001$ for both) and sodium nitroprusside ($p\leq0.002$ for both). Endothelium-dependent and endothelium-independent vasodilatation was similar between the two groups of women (p=0.6 and p=0.8 respectively, two-way ANOVA with repeated measures; Table 2).

TABLE 2. ABSOLUTE FOREARM BLOOD FLOW RESPONSES TO ENDOTHELIUM-DEPENDENT AND INDEPENDENT VASODILATORS

	Healthy non-pregnant women				Healthy pregnant women			
Bradykinin pmol min^{-1}	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
FBF mL^{-1} 100 mL^{-1} min^{-1}								
Non-infused arm	2.0±0.2	2.0±0.3	1.8±0.3	2.0±0.3	3.1±0.6	3.2±0.6	3.6±0.7	3.3±0.6
Infused arm	2.8±0.4	10.1±1.0	13.2±1.2	19.1±1.9*	3.9±0.6	11.2±1.7	15.3±2.5	18.7±2.4*
	Healthy non-pregnant women				**Healthy pregnant women**			
Sodium Nitroprusside μg min^{-1}	**0**	**2**	**4**	**8**	**0**	**2**	**4**	**8**
FBF mL^{-1} 100 mL^{-1} min^{-1}								
Non-infused arm	2.4±0.4	2.4±0.5	2.3±0.4	2.1±0.4	2.9±1.1	3.1±1.2	2.7±1.0	4.3±1.2
Infused arm	3.7±0.9	10.6±1.2	13.1±1.4	16.0±1.9*	4.0±1.4	10.6±2.5	11.2±2.6	14.8±3.2*

Data expressed as mean±SEM. * $p<0.0001$ for bradykinin, * $p=0.002$ and $p<0.0001$ for sodium nitroprusside (pregnant and non-pregnant groups respectively) one-way ANOVA.
FBF - forearm blood flow; SEM - standard error of the mean; ANOVA - analysis of variance.

3.3.3 FIBRINOLYTIC FUNCTION

Baseline plasma PAI-1 antigen concentrations were higher in pregnant women (77.1±12.4 *versus* 21.5±9.8 $ngmL^{-1}$ in non-pregnant women, p=0.004, unpaired Student's *t*-test; Table 3) whilst apparently higher plasma t-PA antigen concentrations did not achieve statistical significance (13.5±2.4 *versus* 7.5±2.0 $ngmL^{-1}$ respectively, p=0.10, unpaired Student's *t*-test). Consistent with this, t-PA/PAI-1 ratios (0.2±0.1 *versus* 0.6±0.1, p=0.02) and plasma t-PA activity concentrations (0.17±0.02 *versus* 0.58±0.06 IU mL^{-1}/mL, p<0.0004, unpaired Student's *t*-test) were lower in pregnant women.

Bradykinin caused a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused arm of both pregnant and non-pregnant women ($p \leq 0.005$ for all; Table 3). The increase in t-PA activity was greater in the non-pregnant women (p=0.02 *versus* pregnant, two-way ANOVA; Figure 16). Bradykinin increased the net release of t-PA antigen and activity in both pregnant women and non-pregnant women (p<0.05 for all; Table 3). Both net release of active t-PA and plasma t-PA/PAI-1 ratios were markedly reduced in pregnant women (p<0.05 for both; Table 3 and Figure 17). Compared with the non-pregnant women, the area under the curve for net active t-PA release was reduced by 36% in the pregnant women.

TABLE 3. PLASMA T-PA AND PAI-1 ANTIGEN AND ACTIVITY CONCENTRATIONS IN BOTH FOREARMS

	Healthy non-pregnant women (n=14)				Healthy pregnant women (n=6)			
Bradykinin Infusion (pmol min^{-1})	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
t-PA Antigen, ng mL^{-1}								
Non-infused arm	7.45±2.0	7.6±2.0	8.0±2.3	9.0±2.8	13.5±2.4	13.6±2.6	14.6±3.0	15.8±3.2
Infused arm	6.95±2.0	8.6±2.0	12.2±2.5	16.0±3.0*	12.3±2.9	13.4±2.8	14.4±2.5	20.0±2.2*
t-PA Activity IU mL^{-1}								
Non-infused arm	0.58±0.06	0.6±0.08	0.8±0.09	1.1±0.14	0.17±0.02	0.26±0.06	0.43±0.2	0.53±0.12
Infused arm	0.5±0.06	1.6±0.19	2.8±0.4	4.3±0.6*	0.51±0.32	0.51±0.1	1.3±0.15	2.4±0.31*
PAI-1 Antigen, ng mL^{-1}								
Non-infused arm	21.5±9.8	23.2±10.3	23±10	26.9±10	77.1±12.4	71.8±6.3	80.5±11.4	78.5±5.8
Infused arm	19.2±7.7	18±7.3	19±7	19.8±7.4	72.6±9.0	87.1±16.3	82.8±14	82.9±13
PAI-1 Activity, ng mL^{-1}								
Non-infused arm	2.1±1.8	1.4±1.0	1.1±0.9	1.1±0.8	5.7±1.4	6.1±1.7	6.2±1.7	5.8±1.6
Infused arm	1.36±1.0	1.31±1.0	1.9±1.7	1.1±0.9	5.7±1.5	5.9±1.7	5.8±1.7	6.6±2.5
Net t-PA Antigen release ng 100 mL^{-1} of tissue min^{-1}	-0.05±0.8	12.2±3.19	62.3±13	150.3±32.6	-2.8±3.03	0.07±8.0	6.63±15.8	101.6±59
Net t-PA Activity release IU 100 mL^{-1} of tissue min^{-1}	0.17±0.07	8.8±1.3	24.7±2.7	60.8±11.2†	0.85±1.0	3.4±2.0	17.2±7.4	41.3±13.1†‡

Data expressed as mean±SEM for 6 pregnant and 14 non-pregnant subjects. Fibrinolytic data not available or incomplete for 3 pregnant and 3 non-pregnant women due to technical difficulties with cannulation during study.

*$p \leq 0.005$, dose-dependent increase in plasma t-PA antigen and activity. †$p<0.05$, increase in net release of t-PA antigen and activity. ‡$p<0.05$, net release of active t-PA was reduced in pregnant women. t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type 1; SEM - standard error of the mean.

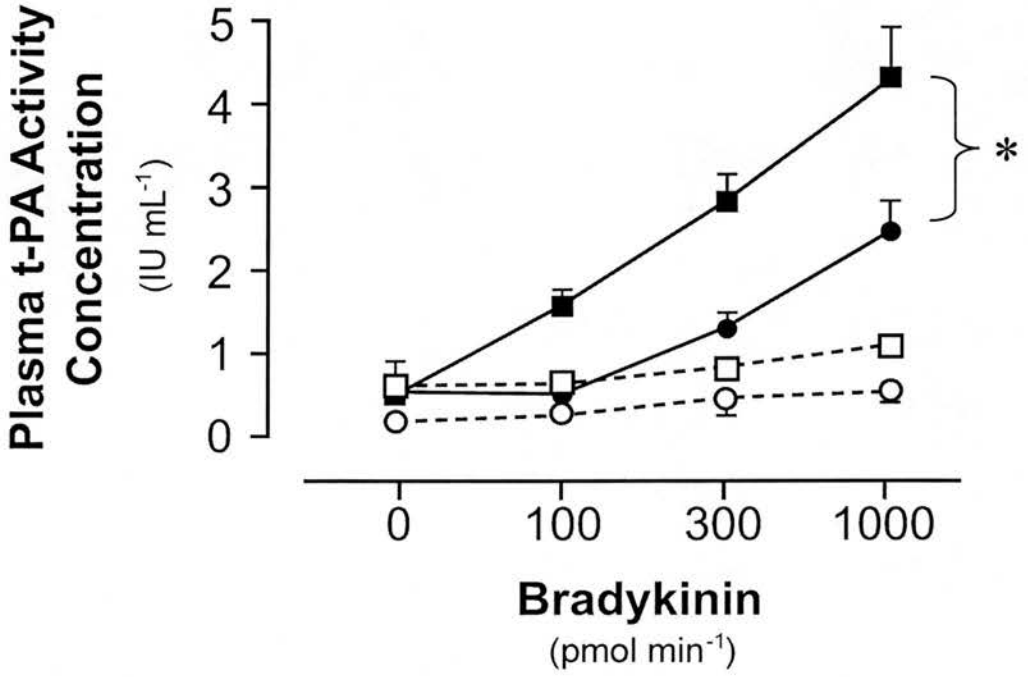

Figure 16. Plasma tissue plasminogen activator (t-PA) activity concentrations in the infused (solid symbols and lines) and non-infused (open symbols and dashed lines) arms of pregnant (n=6, circles) and non-pregnant (n=14, squares) women during intra-arterial bradykinin infusion. Plasma t-PA activity increased in the infused arm of both pregnant and non-pregnant women ($p<0.001$ for both, one-way analysis of variance (ANOVA) but was greater in non-pregnant women (*$p=0.02$ *versus* pregnant women, two-way ANOVA).
IU - international units.

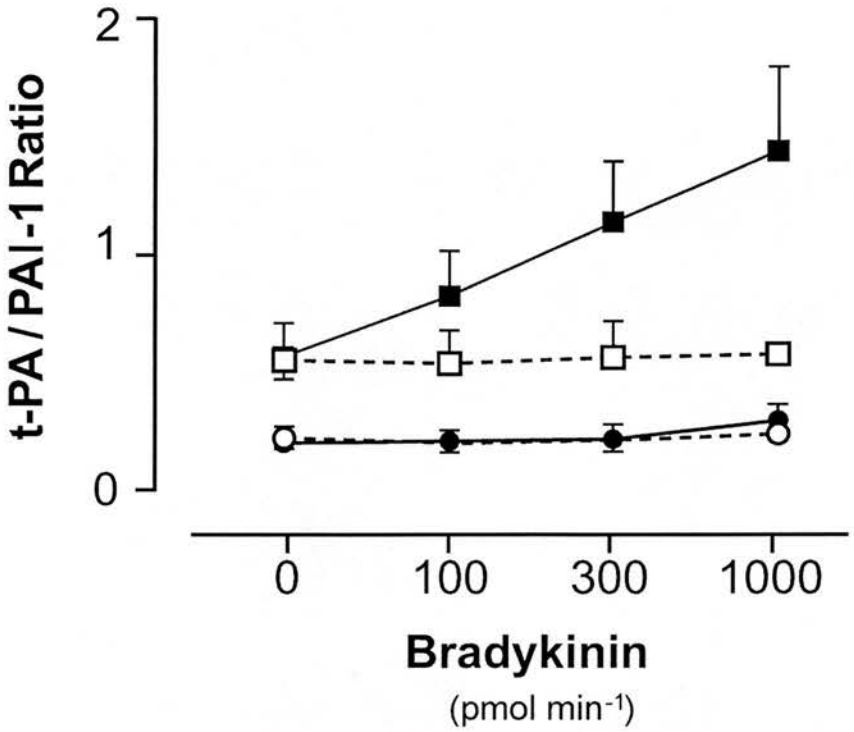

Figure 17. Ratio of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) in the infused (solid symbols and lines) and non-infused (open symbols and dashed lines) arms of pregnant (n=6, circles) and non-pregnant (n=14, squares) women during intra-arterial bradykinin infusion. Plasma t-PA/PAI-1 ratios were markedly lower in pregnant women (p=0.04, *versus* non-pregnant women, two-way analysis of variance (ANOVA)).

3.4 DISCUSSION

In the third trimester of pregnancy, these results demonstrate that there is marked perturbation of the fibrinolytic system. There was a marked increase in basal plasma PAI-1 concentrations leading to a reduction in t-PA activity and t-PA/PAI-1 ratios, consistent with previous work [Koh *et al*, 2002; Coolman *et al*, 2006]. For the first time, acute endothelial t-PA release has been assessed in pregnant women. Despite normal vasomotor responses, there was a marked reduction in bradykinin-induced release of active t-PA that was primarily attributable to an excess of PAI-1. This leads to the conclusion that elevated plasma PAI-1 concentrations and inadequate active t-PA release contribute to the pro-thrombotic consequences of pregnancy.

3.4.1 PERTURBATIONS OF FIBRINOLYSIS IN PREGNANCY

Pregnancy is associated with a marked elevation in plasma PAI-1 concentrations that, in this study, were 4- to 5-fold higher than age-matched non-pregnant women and comparable to concentrations found in previous studies [Chappell *et al*, 2002]. Although there appeared to be a modest concomitant rise in t-PA antigen, this was unable to compensate for the dominance of PAI-1 as demonstrated by the adverse effect on the ratio of t-PA to PAI-1. Thus, whilst being able to stimulate the release of substantial amounts of t-PA from the endothelium using intra-arterial bradykinin, the overwhelming effect of PAI-1 markedly inhibited the plasma activity of both basal and stimulated t-PA. This marked shift in fibrinolytic potential, and the associated dramatic reduction in active t-PA, will have major pro-thrombotic consequences for pregnant women.

During pregnancy, PAI-1 is produced by placental vasculature, trophoblast and decidua in addition to endothelium and activated platelets [Ginsburg *et al*, 1986; Schatz and Lockwood, 1993; Fitzpatrick and Graham, 1998]. The affinity of PAI-1 for t-PA is 1000 times greater than that of PAI-2, which is also produced by the placenta [Vassalli *et al*, 1991]. The placenta therefore plays a major role in regulating the fibrinolytic balance during pregnancy. In pregnancies complicated by placental dysfunction, the reduction in fibrinolytic capacity is even more pronounced [Greer, 2003]. For example, in pre-eclampsia, increased placental synthesis of PAI-1 [Estelles *et al*, 1998] is associated with aberrant fibrin deposition and the development of vaso-occlusive lesions within the placenta [Ma *et al*, 2002]. Moreover, the hypofibrinolytic polymorphic gene mutations of PAI-1, for example the 4G/4G and 4G/5G mutations, which cause an increase in placental PAI-1 gene expression and plasma levels, are independent risk factors for the development of pre-eclampsia [Yamada *et al*, 2000; Glueck *et al*, 2001].

Pregnancy is also associated with an increased risk of systemic thrombotic events including thromboembolism and myocardial infarction. Although rare (6 per 100,000 pregnancies), the age-adjusted incidence of acute myocardial infarction is increased 4-fold by pregnancy and is associated with a significant mortality rate [James *et al*, 2006]. With increasing maternal age, the overall incidence of myocardial infarction is rising (30 per 100,000 in those over 40 years) and is likely to represent an increasing patient group in contemporary cardiovascular practice.

Acute myocardial infarction in women has several features that distinguish it from those seen in men [Davies, 2000]. In 50% of women, myocardial infarction results from plaque erosion rather than rupture and, in some instances, endothelial erosion and coronary thrombosis can occur in the absence of atherosclerosis [Arbustini *et al*, 1999]. Thus, thrombosis and pro-thrombotic states are more likely to be implicated in the pathogenesis of acute myocardial infarction in women and this may be mediated through changes in fibrinolytic function.

This current study has demonstrated a reduction of bradykinin-induced release of active t-PA and t-PA/PAI-1 ratio in normal pregnancy compared to non-pregnant women. This is consistent with a study by Stegnar *et al* [Stegnar *et al*, 1993], that demonstrated reduced t-PA activity in pregnant, compared with non-pregnant, women after the venous occlusion test. This finding also mirrors that of a previous study in which it was demonstrated that current smokers had elevated basal plasma PAI-1 concentrations, that were associated with reduced basal and stimulated active t-PA, compared to non-smokers [Newby *et al*, 2001]. Coronary thrombosis [Doll *et al*, 2004] is one of the major causes of sudden cardiac death in both smokers [Burke *et al*, 1997] and myocardial infarction during pregnancy [Davies, 2000]. The alteration in fibrinolytic function that has been demonstrated may therefore provide a common pathogenic mechanism underlying coronary thrombosis in both patient populations. Moreover, it may in part explain the strong association of myocardial infarction with hypertension during pregnancy. Superimposing the added stress of hypertension on blood vessels already weakened by the haemodynamic stresses of

pregnancy may augment the reduction in t-PA and t-PA/PAI-1 ratio in response to endothelial damage [Hrafnkelsdottir *et al*, 2004].

Bradykinin is a powerful stimulus for endothelial t-PA release and has been used in this study and by other investigators to assess this aspect of endothelial function [Brown *et al*, 1999; Brown *et al*, 2000; Mills *et al*, 2007]. Bradykinin is not only an inflammatory mediator but is also released during the contact phase of coagulation when high-molecular weight kininogen is cleaved by kallikrein to produce a disulphide-linked light and heavy chain [Schiffman *et al*, 1980; Reddigari and Kaplan, 1988]. This liberation of bradykinin may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits thrombus formation within the vascular lumen when localised endothelial denudation occurs. Indeed, bradykinin may also contribute to the regulation of basal release of t-PA and that associated with angiotensin-converting enzyme inhibition [Pretorius *et al*, 2003]. Thus, whilst bradykinin was used in this study as a physiological tool to probe the effects of pregnancy, it may also have a pathophysiological role in the pathway of acute t-PA release as has been demonstrated in cardiovascular disease in non-pregnant patients [Cruden and Newby, 2005].

3.4.2 VASOMOTOR FUNCTION

The study design allowed the assessment of peripheral vasomotor function of women in pregnancy in addition to the dynamic fibrinolytic capacity. Whilst not the primary aim, this study demonstrates that, despite the major hyperdynamic changes of pregnancy, endothelial and vascular smooth muscle vasomotor function was

preserved and comparable to normal non-pregnant women. Consistent with these findings, Anumba and colleagues [Anumba *et al*, 1999b] have previously reported unaltered nitric oxide sensitivity and endothelium-independent vasodilatation during pregnancy. However, they did find a modest impairment of endothelium-dependent vasodilatation to serotonin. Whether this reflects differential alterations in signalling pathways, or a true alteration in endothelial function, remains to be determined. This highlights the importance of assessing different aspects of vascular function in order to determine the overall effect of pregnancy on the vasculature.

3.5 SUMMARY

In conclusion, pregnancy is associated with major perturbations of endogenous fibrinolytic capacity that is due an overwhelming increase in plasma PAI-1 concentrations and an inadequate release of active t-PA. These pro-thrombotic effects may, in part, explain the increased risk of arterial and venous thrombosis both systemically and locally within the foeto-placental unit in pregnant women.

CHAPTER 4

THE INFLUENCE OF THE MENSTRUAL CYCLE, HEALTHY PREGNANCY AND PRE-ECLAMPSIA ON PLATELET AND MONOCYTE ACTIVATION

4.1 INTRODUCTION

Inflammation and haemostasis underpin many reproductive processes including menstruation, healthy and complicated pregnancies. Circulating platelets and their interactions with monocytes and the endothelium play a key role in mediating thrombotic and inflammatory events in health and disease. Activated platelets express P-selectin on their cell surface and form aggregates with leucocytes, predominately monocytes [Sarma *et al*, 2002]. The formation of platelet-monocyte aggregates has important functional consequences. These include the expression and release of cytokines, chemokines, adhesion molecules and tissue factor by monocytes. In addition, monocyte adhesion and subsequent interaction with the endothelium is promoted.

Platelets are extremely susceptible to *ex vivo* activation by sampling and handling methods. The use of flow cytometry is a sensitive technique to evaluate *in vivo* platelet and monocyte activation in whole blood. Using this technique, the expression of platelet surface P-selectin, monocyte surface CD40 and CD11b and the formation of circulating platelet-monocyte aggregates can be quantified. These are considered highly sensitive markers of platelet [Michelson *et al*, 2001] and monocyte activation [Shi and Simon, 2006].

Establishing whether there is variation in platelet and monocyte activation during the menstrual cycle, in normal and pre-eclamptic pregnancies may further our

understanding of pregnancy and its complications and perhaps inform the temporal link between thromboembolic disease and reproductive processes.

The aim of this research was to determine the influence of the menstrual cycle, normal and pre-eclamptic pregnancies on platelet and monocyte activation using both cellular (platelet-monocyte aggregates, platelet surface P-selectin expression, monocyte CD40 and CD11b expression) and soluble (soluble P-selectin and soluble CD40L) markers of activation.

4.2 METHODS

All materials, reagents and cell lines used are detailed in Appendix 1.

4.2.1 SUBJECT RECRUITMENT

Women were identified, recruited and consented to this study (study 2, Reference number 05/S1104/48), as described in section 2.1.

Non-pregnant women

Healthy pre-menopausal non-smoking nulliparous women (n=16) with at least a 2-month history of normal regular menstrual cycles were recruited to the study. Exclusion criteria included the use of hormonal contraception, current or past heavy menstrual bleeding or dysmennorhoea, current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation as detailed in section 2.1.2.

Pregnant women

Healthy, non-smoking primigravida women with an uncomplicated pregnancy (n=30) or with pre-eclampsia (n=16) were recruited in the first trimester of pregnancy or at diagnosis respectively. Women were given a minimum of 24 hours to consider participating in the study. Exclusion criteria are detailed in section 2.1.3.

4.2.2 VISIT SCHEDULE

Women abstained from alcohol, caffeine and tobacco for 12 hours and fasted for 4 hours prior to attendance. Blood pressure and heart rate were recorded in duplicate using an automated sphygmomanometer following a 15 minute left-lateral or supine rest for pregnant subjects (at every visit) and non-pregnant (at first visit, Day (D) 1-3) respectively. Height and weight were also measured to allow calculation of BMI. Women with pre-eclampsia continued to take their antihypertensive medication as prescribed. This is detailed in section 4.3.1.

Non-pregnant women

Women attended for four visits during a single menstrual cycle (D 1-3 (early follicular), D 6-8 (mid-follicular)), D 13-15 (peri-ovulatory) and D 20-22 (mid-luteal)).

Pregnant women

Women with uncomplicated pregnancies attended for four visits during pregnancy at 16, 24, 32 and 37 weeks and at 7 weeks post-partum. Women with pre-eclampsia attended following diagnosis and at 7 weeks post-partum.

4.2.3 SAMPLE COLLECTION

At each visit, peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle using minimal tourniquet pressure, as described in section 2.4.2. Care was taken to ensure a smooth blood draw as described previously [Harding *et al*, 2007].

Initial processing of all samples occurred within 5 minutes of phlebotomy, with immunolabelling performed at exactly 5 minutes to minimise *ex vivo* platelet activation. Plasma and serum were prepared and stored as described in section 2.4.2 for subsequent analysis of circulating hormones and markers of platelet activation. Following venepuncture, arterial stiffness studies were performed, detailed in Chapter 6.

4.2.4 FLOW CYTOMETRIC ANALYSIS OF PLATELET AND MONOCYTE ACTIVATION

Platelet-monocyte aggregates

Staining was performed on whole blood using directly conjugated monoclonal antibodies, on all samples as detailed in section 2.5.1.

Platelet surface P-selectin expression

Staining was performed on whole blood using directly conjugated monoclonal antibodies, on all samples as detailed in section 2.5.2.

Monocyte CD40 and CD11b expression

Staining was performed on whole blood using directly conjugated monoclonal antibodies, on all samples as detailed in section 2.5.3.

4.2.5 Measurement of soluble markers of platelet activation

Circulating P-selectin and soluble CD40L concentrations were measured in plasma using commercially available enzyme-linked immunosorbant assays as described in sections 2.8.1 and 2.8.2 respectively.

4.2.6 Measurement of circulating hormones

Serum concentrations of oestradiol and progesterone were measured in all women. Lutenizing hormone and follicle-stimulating hormone were also measured in the healthy non-pregnant women. All hormones were measured by automated chemiluminescent microparticle assays as described in section 2.9.1-4.

4.2.7 Statistical analysis

The distribution of data was determined using the Kolmogorov-Smirnov test for normality. Continuous variables are reported as mean±SEM for parametric data. Statistical analyses were performed using paired and unpaired two-tailed Student's *t*-tests. For multiple comparisons, one-way ANOVA with repeated measures was used. All calculations were performed using GraphPad Prism, (GraphPad Software, USA). Statistical significance was taken at 5%.

4.3 RESULTS

4.3.1 SUBJECT CHARACTERISTICS

Non-pregnant women

In six of sixteen non-pregnant women, ovulation could not be confirmed leaving a final study group of ten women with a confirmed regular ovulatory cycle (cycle length 28±0.4 days; luteal phase serum progesterone >30 nmol/L). All women (age, 31.4±2.0 years) were of normal body composition (BMI, 23.4±0.7 kg/m^2). Baseline systolic and diastolic blood pressures and heart rate, measured at D 1-3 of the cycle, were 110±2 mmHg, 67±2 mmHg and 60±2 bpm, respectively.

Pregnant subjects

In the healthy pregnant group, four of thirty women developed complications in their pregnancy, for example intra-uterine growth restriction, pre-term labour or pre-eclampsia and a further five were unable to complete the full schedule of visits, leaving a final study group of twenty-one women for analysis. In the pre-eclamptic group, women were categorised according to gestation at presentation, into pre-term (n=8; mean 30 (range 24-34 weeks)) and term (n=8; mean 38 (range 36-40 weeks). Women with pre-eclampsia were delivered earlier than women with uncomplicated pregnancies and had offspring of lower birth weight (Table 4).

There was no difference in first trimester blood pressures between women with healthy and pre-eclamptic pregnancies (113±2/69±2 mmHg *versus* 117±3/73±2 mmHg, $p>0.9$). As expected women, with pre-term and term pre-eclampsia had higher blood pressures at the time of study compared to those women with uncomplicated pregnancies, at similar gestations (pre-term pre-eclampsia, 30 weeks (n=8) at presentation *versus* healthy pregnancy, 32 weeks (n=21) 140±2/87±2 mmHg *versus* 112±1/69±1 mmHg respectively; term pre-eclampsia, 38 weeks (n=8) at presentation *versus* healthy pregnancy, 37 weeks (n=21) 146±2/95±2 mmHg *versus* 116±1/75±1 mmHg respectively; all $p<0.002$). The pre-eclamptic women were no longer hypertensive at the post-partum visit, but still had higher blood pressures than the women with uncomplicated pregnancies (pre-eclampsia post-partum (n=16) 123±3/77±2 mmHg *versus* healthy pregnancy post-partum (n=21) 113±1/69±1 mmHg; both $p<0.002$).

Medication use

In women with pre-term pre-eclampsia, five were taking regular labetalol and nifedipine, two were taking regular methyldopa and nifedipine, and one was receiving no anti-hypertensive therapy. Seven of these women received antenatal betamethasone. In women with term pre-eclampsia, one was taking regular labetalol with the remaining seven women not receiving anti-hypertensive therapy. None received antenatal betamethasone. Post-partum, out of the original sixteen women who had developed pre-eclampsia, only three women were taking labetalol, and one, methyldopa.

TABLE 4. BASELINE CHARACTERISTICS OF STUDY PARTICIPANTS

	Healthy non-pregnant group (n=10)	Healthy pregnant group (n=21)	Pre-eclamptic group (n=16)	
			Pre-term (n=8)	Term (n=8)
Age (years)	31±2	30±1	28±3	30±2
Height (m)	1.70±0.02	1.64±0.02*	1.63±0.02*	1.62±0.02*
Weight (Kg)	68±3	72±4	64±4	75±4
Body Mass Index (kg/m2)	23±1	26±1	24±2	29±1*
Gestation at delivery (weeks)	N/A	41±0.3	31±1.4‡	39±0.4†
Birth weight (g)	N/A	3447±117	1366±192‡	3231±252

Data expressed as mean±SEM. Body mass index was recorded during the first trimester for pregnant subjects.
* p=0.0009 *versus* non-pregnant group, † p=0.0005 *versus* healthy pregnant group, ‡ p≤0.0001 *versus* healthy pregnant and term pre-eclamptic groups (two-tailed unpaired Student's *t*-tests).
N/A - not applicable; SEM - standard error of the mean.

4.3.2 Effect of the menstrual cycle on platelet activation

To investigate the effect of cyclical variation in ovarian hormones on platelet activation: platelet-monocyte aggregates, platelet surface P-selectin expression, plasma soluble P-selectin and soluble CD40L concentrations were measured longitudinally over a single menstrual cycle in healthy non-pregnant women. None of these measures of platelet activation varied during the menstrual cycle ($p>0.08$ for all, one-way ANOVA with repeated measures; Figures 18, 19, 20 and 21). There was no correlation between platelet-monocyte aggregates, surface or soluble P-selectin or soluble CD40L and circulating concentrations of oestrogen or progesterone at any stage of the cycle ($p>0.05$ for all).

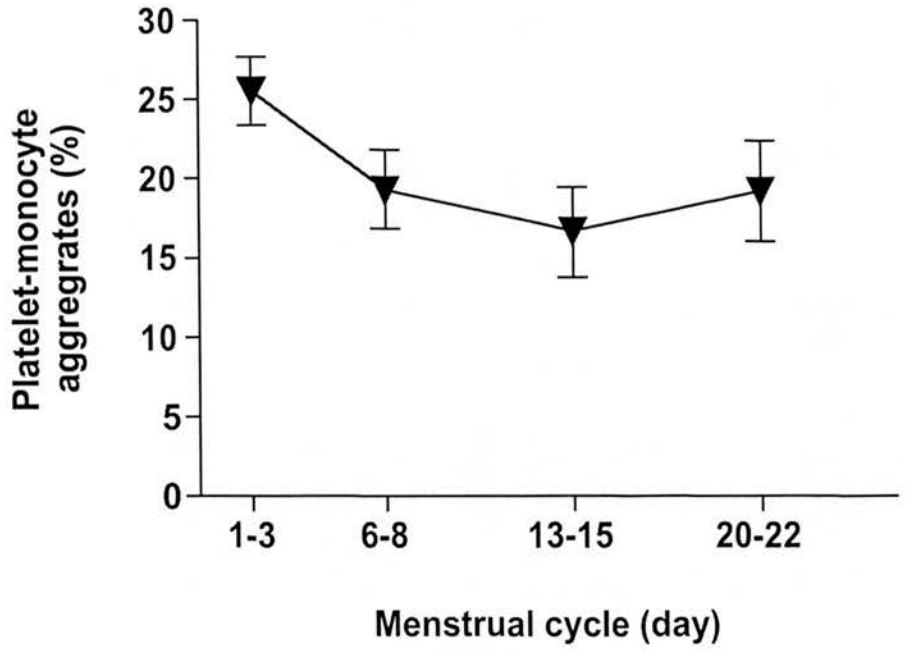

Figure 18. Platelet-monocyte aggregates do not vary during the menstrual cycle, ($p=0.09$, one-way ANOVA with repeated measures). Data (non-pregnant women, $n=10$) expressed as mean±SEM. ANOVA - analysis of variance; SEM - standard error of the mean.

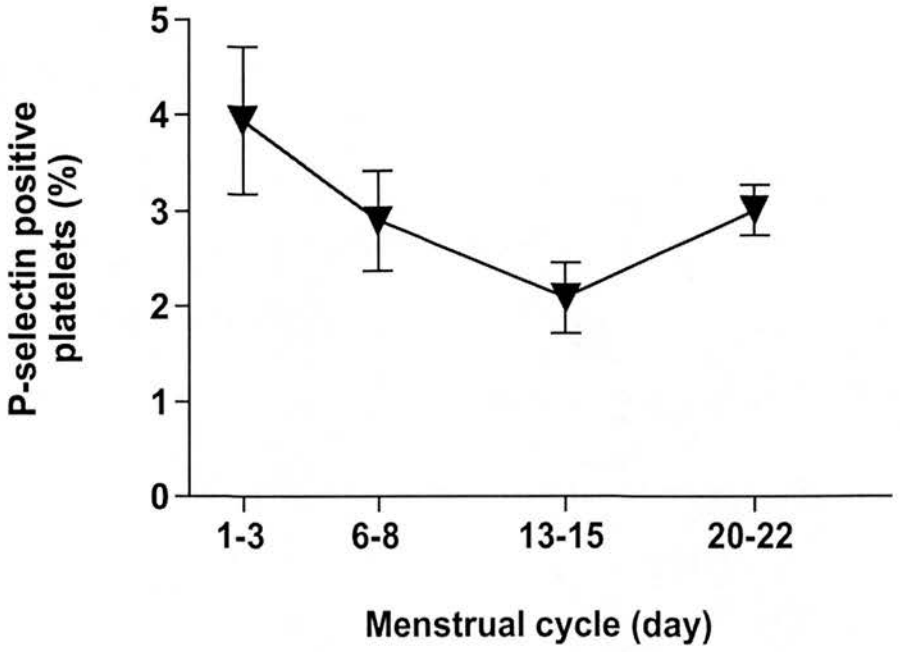

Figure 19. Platelet surface expression of P-selectin does not vary during the menstrual cycle, (p=0.08, one-way ANOVA with repeated measures). Data (non-pregnant women, n=10) expressed as mean±SEM. ANOVA - analysis of variance; SEM - standard error of the mean.

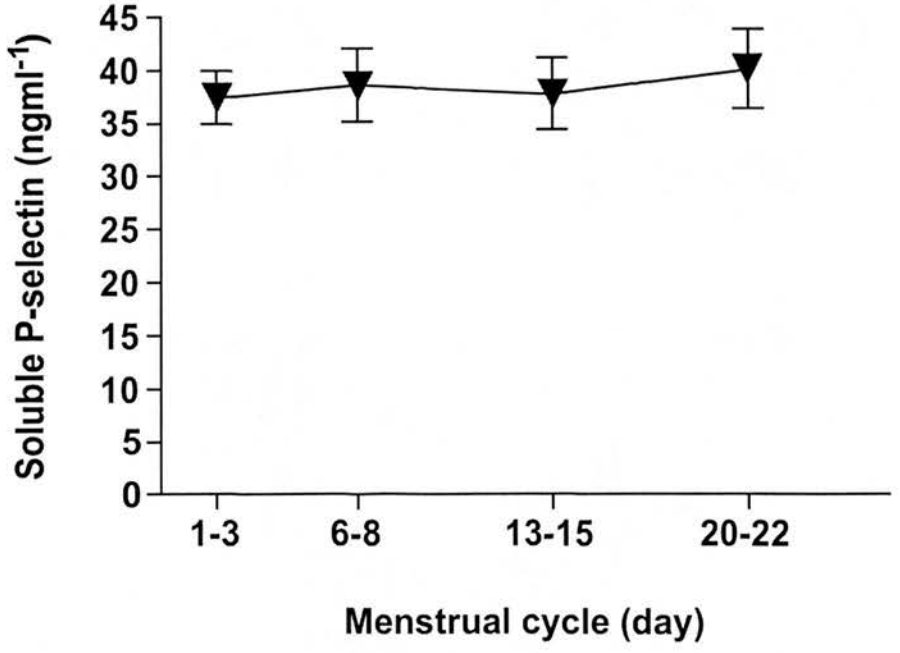

Figure 20. Plasma concentrations of soluble P-selectin ($ngmL^{-1}$) do not vary during the menstrual cycle, (p=0.7, one-way ANOVA with repeated measures). Data (non-pregnant women, n=10) expressed as mean±SEM. ANOVA - analysis of variance; SEM - standard error of the mean.

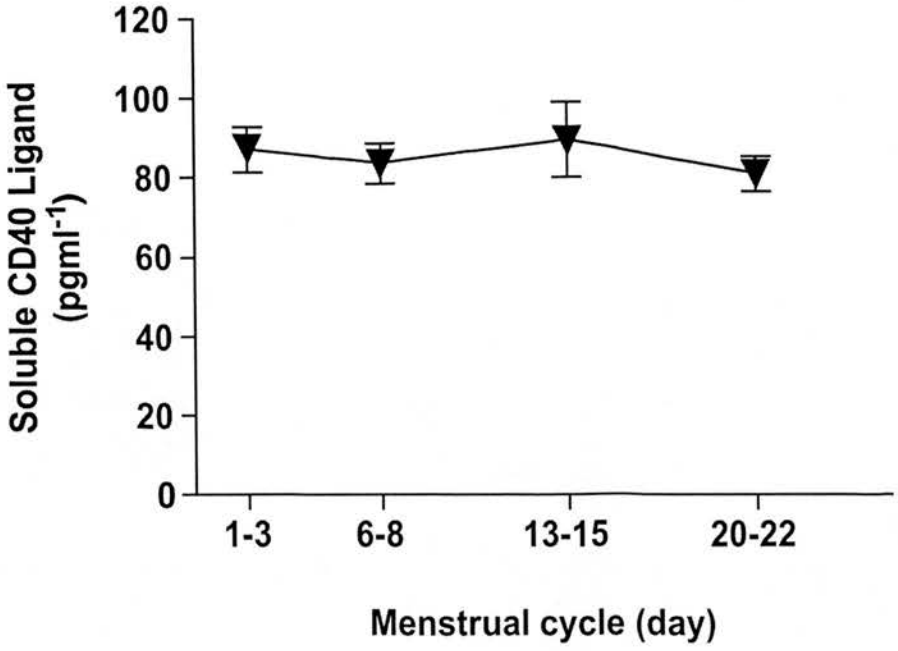

Figure 21. Plasma concentrations of soluble CD40 ligand ($pgmL^{-1}$) do not vary during the menstrual cycle, ($p=0.6$, one-way ANOVA with repeated measures). Data (non-pregnant women, $n=10$) expressed as mean±SEM. CD - cluster of differentiation; ANOVA - analysis of variance; SEM - standard error of the mean.

4.3.3 Effect of the menstrual cycle on monocyte activation

To investigate the effect of cyclical variation in ovarian hormones on monocyte activation, monocyte CD40 and CD11b expression were measured longitudinally over a single menstrual cycle in healthy non-pregnant women. Neither of these measures of monocyte activation varied during the menstrual cycle ($p>0.3$ for both, one-way ANOVA with repeated measures; Figures 22 and 23). There was no correlation between monocyte CD40 or CD11b expression and circulating concentrations of oestrogen or progesterone at any stage of the cycle ($p>0.05$ for all).

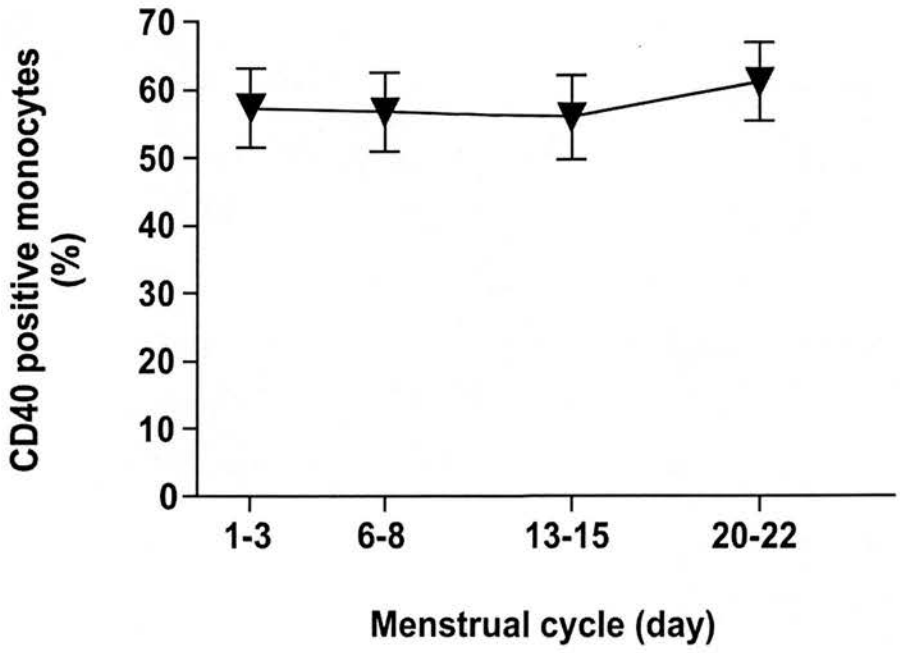

Figure 22. Monocyte CD40 expression does not vary through the menstrual cycle, (p=0.3, one-way ANOVA with repeated measures). Data (non-pregnant women, n=10) expressed as mean±SEM.
CD - cluster of differentiation; ANOVA - analysis of variance; SEM - standard error of the mean.

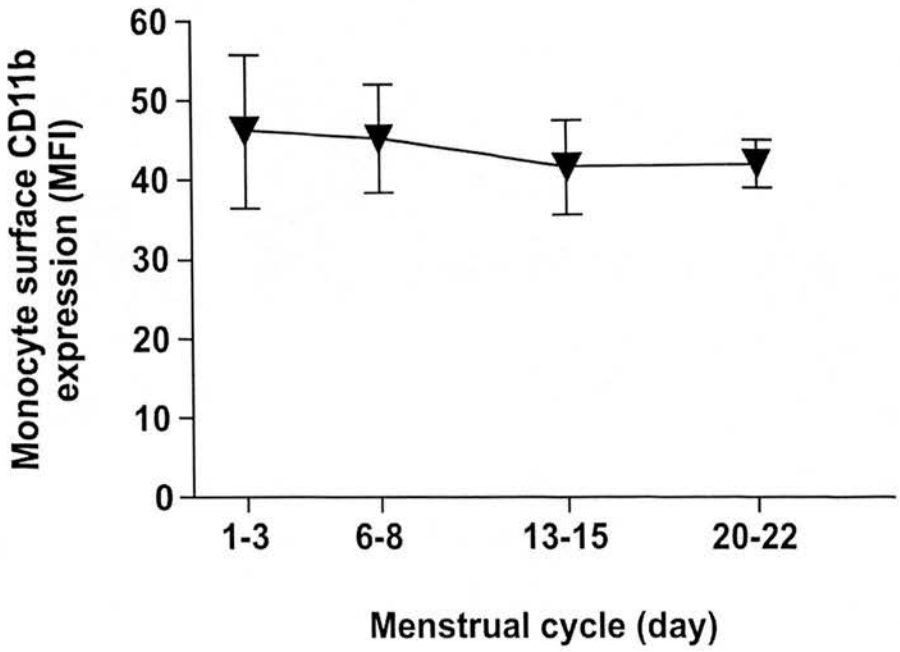

Figure 23. Monocyte CD11b expression (MFI), does not vary through the menstrual cycle, (p=0.8, one-way ANOVA with repeated measures). Data (non-pregnant women, n=10) expressed as mean±SEM. CD - cluster of differentiation; MFI - mean fluorescent intensity; ANOVA - analysis of variance; SEM - standard error of the mean.

4.3.4 Effect of pregnancy on platelet activation

To investigate the effect of healthy pregnancy on platelet activation: platelet-monocyte aggregates, platelet surface P-selectin expression, plasma soluble P-selectin and CD40L concentrations were quantified longitudinally during healthy pregnancy and compared to the mean of the values obtained across the menstrual cycle from the ten non-pregnant women.

Platelet-monocyte aggregates did not vary significantly during healthy pregnancy (p=0.28, one-way ANOVA with repeated measures; Figure 24).

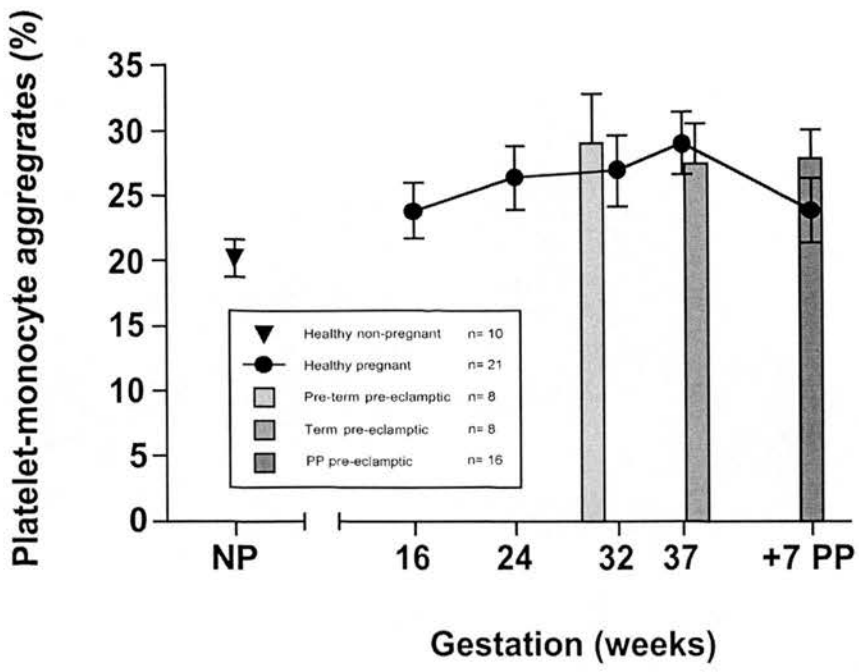

Figure 24. Influence of normal and pre-eclamptic pregnancy on platelet-monocyte aggregates. Platelet-monocyte aggregates increased by 24 weeks gestation, compared to the non-pregnant women ($p=0.02$, two-tailed, unpaired Student's *t*-test). Platelet-monocyte aggregates did not vary with gestation ($p=0.28$, one-way ANOVA with repeated measures), but did decrease post-partum ($p=0.02$, 37 weeks *versus* post-partum). Platelet-monocyte aggregates were not significantly different in women with pre-eclampsia. Data expressed as mean±SEM.
NP - non-pregnant; PP - post-partum; ANOVA - analysis of variance; SEM - standard error of the mean.

Platelet surface and plasma soluble P-selectin increased with gestation during healthy pregnancy ($p<0.0001$, one-way ANOVA with repeated measures; Figures 25 and 26). Compared to the non-pregnant women, platelet-monocyte aggregates, platelet surface P-selectin expression and plasma soluble P-selectin concentrations were all higher in healthy pregnancy by 24 weeks gestation (26.4±2.5% *versus* 20.2±1.4%; 4.7±0.6% *versus* 3.0±0.3% and 47.1±3.0 $ngmL^{-1}$ *versus* 38.6±1.6 $ngmL^{-1}$, respectively; $p\leq0.02$ for all, two-tailed unpaired Student's *t*-test). Platelet-monocyte aggregates, platelet surface P-selectin expression and plasma soluble P-selectin

concentrations all decreased post-partum ($p \leq 0.02$ for all, two-tailed paired Student's *t*-test), although platelet surface P-selectin and plasma soluble P-selectin concentrations remained elevated 7 weeks post-partum compared to the non-pregnant women (4.0±0.4% *versus* 3.0±0.3% and 49.6±4.0 $ngmL^{-1}$ *versus* 38.6±1.6 $ngmL^{-1}$, respectively; $p<0.03$ for both, two-tailed unpaired Student's *t*-test).

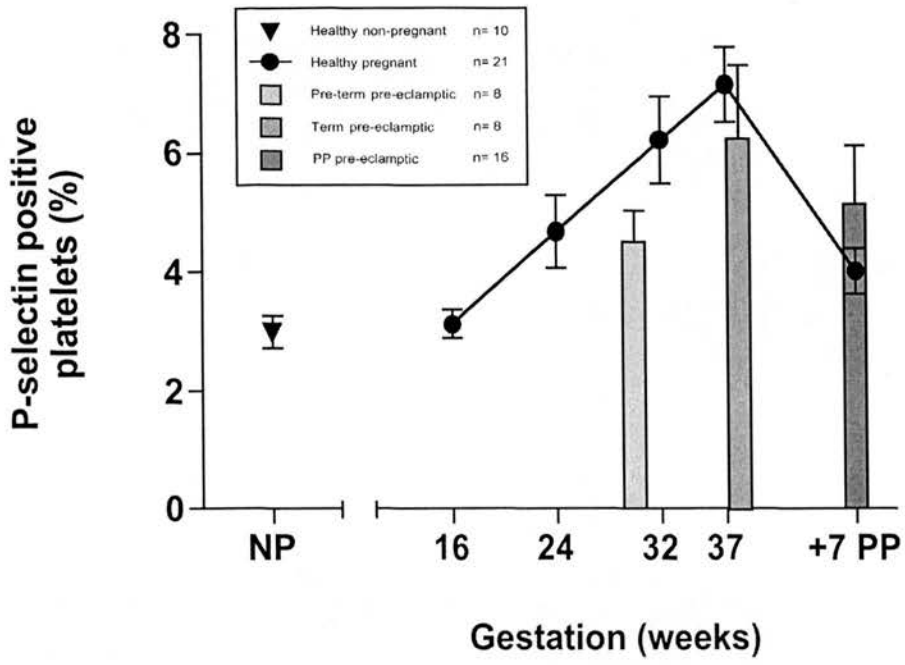

Figure 25. Influence of normal and pre-eclamptic pregnancy on platelet surface P-selectin expression. Platelet surface P-selectin expression increased by 24 weeks gestation, compared to the non-pregnant women ($p=0.005$, two-tailed unpaired Student's *t*-test), varied with gestation ($p<0.001$, one-way ANOVA with repeated measures), and decreased post-partum ($p<0.001$, 37 weeks *versus* post-partum, two-tailed paired Student's *t*-test). Expression was not significantly different in pre-eclampsia. Data expressed as mean±SEM.
NP - non-pregnant; PP - post-partum; ANOVA - analysis of variance; SEM - standard error of the mean.

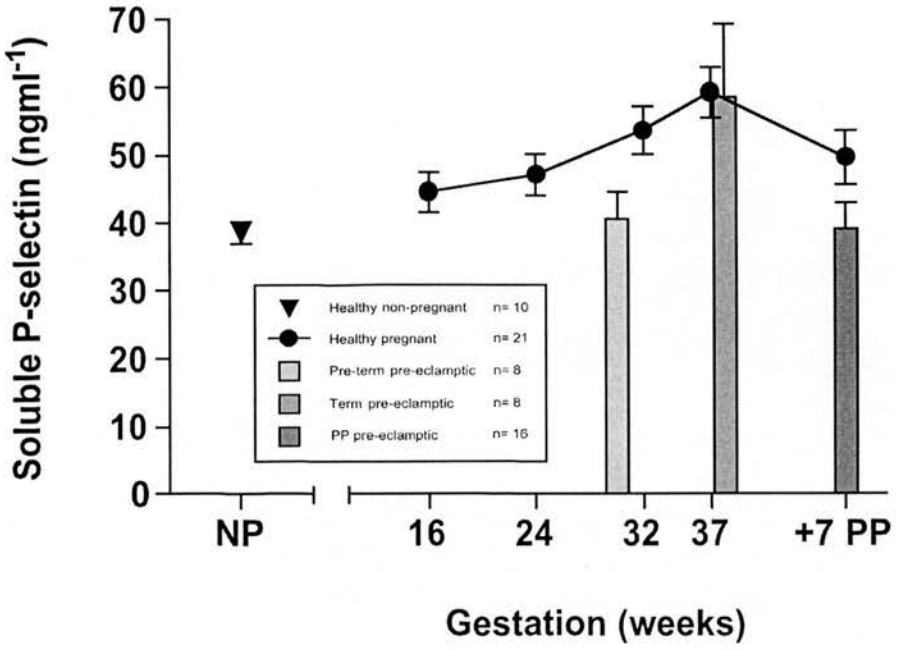

Figure 26. Influence of normal and pre-eclamptic pregnancy on the concentration of soluble P-selectin in plasma. Plasma concentrations of soluble P-selectin ($ngmL^{-1}$) increased by 24 weeks gestation, compared to the non-pregnant women ($p=0.007$, two-tailed unpaired Student's *t*-test), varied with gestation ($p<0.001$, one-way ANOVA with repeated measures), and decreased post-partum ($p<0.02$, 37 weeks *versus* post-partum, two-tailed paired Student's *t*-test). Plasma concentrations were not significantly different in pre-eclampsia. Data expressed as mean±SEM.
NP - non-pregnant; PP - post-partum; ANOVA - analysis of variance; SEM - standard error of the mean.

Compared to non-pregnant women, plasma soluble CD40L concentrations were lower in pregnancy at mid gestation (64.8±5.3 $pgmL^{-1}$ *versus* 85.5±3.1 $pgmL^{-1}$, 24 weeks *versus* non-pregnant women, $p=0.0007$, two-tailed unpaired Student's *t*-test; Figure 27). Levels fell from 16 to 24 weeks ($p<0.01$, Bonferroni's post-test) before rising at term. Soluble CD40L concentrations continued to rise post-partum ($p=0.03$, two-tailed paired Student's *t*-test) and were higher than non-pregnant levels at 7 weeks post-partum (101.2±7.6 $pgmL^{-1}$ *versus* 85.5±3.1 $pgmL^{-1}$, 7 weeks post-partum *versus* non-pregnant women, $p=0.03$, two-tailed unpaired Student's *t*-test).

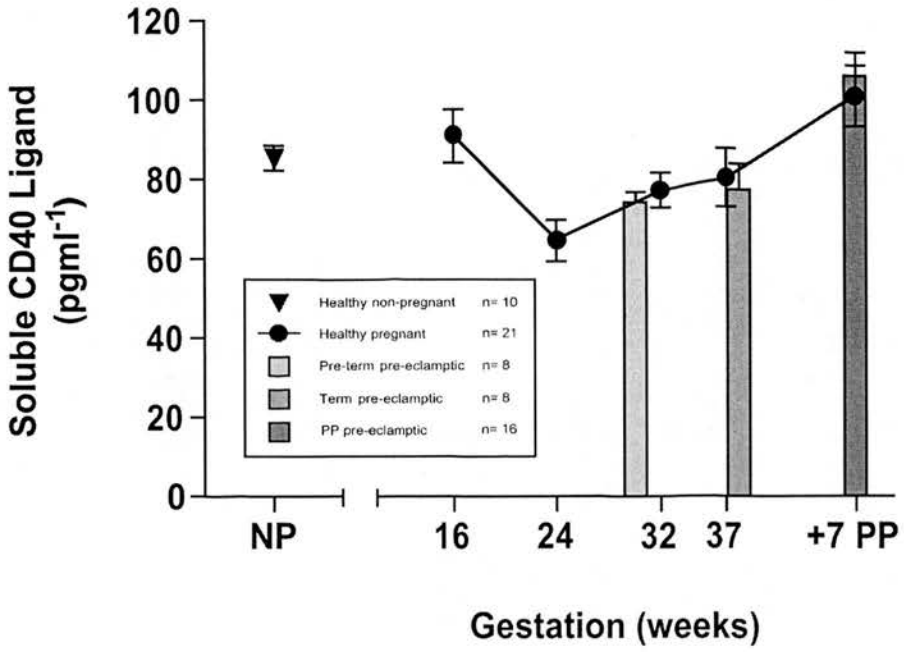

Figure 27. Influence of normal and pre-eclamptic pregnancy on the concentration of soluble CD40 ligand in plasma. Plasma concentrations of soluble CD40 ligand ($pgmL^{-1}$) were lower at 24 weeks than in non-pregnant women ($p=0.0007$, two-tailed unpaired Student's *t*-test) varied with gestation ($p=0.01$, one-way ANOVA with repeated measures), falling between 16 and 24 weeks ($p<0.01$, Bonferroni's post-test) and increased post-partum ($p=0.03$; 37 weeks *versus* post-partum, two-tailed paired Student's *t*-test). Plasma concentrations were not significantly different in pre-eclampsia. Data expressed as mean±SEM.
NP - non-pregnant; PP - post-partum; CD - cluster of differentiation; ANOVA - analysis of variance; SEM - standard error of the mean.

There was no difference in any of the variables measured between pre-term or term pre-eclamptic and healthy pregnant women. Although the post-partum data is combined graphically for the women with pre-eclampsia, there was no difference in any of the variables measured between women with pre-term or term pre-eclampsia at the post-partum visit (two-tailed unpaired Student's *t*-tests; $p>0.05$ for all; Figures 24, 25, 26 and 27).

4.3.6 Effect of pregnancy on monocyte activation

To investigate the effect of healthy pregnancy on monocyte activation; monocyte CD40 and CD11b expression were quantified longitudinally during healthy pregnancy and compared to the mean of the values obtained across the menstrual cycle from the ten non-pregnant women.

Monocyte CD40 expression was lower at 16 weeks in healthy pregnant women, than in non-pregnant women (41.5±3.3% *versus* 57.7±2.8%, p=0.0009, two-tailed unpaired Student's *t*-test), then increased with gestation (p<0.0001, one-way ANOVA with repeated measures) and decreased post-partum (37 weeks *versus* post-partum, 62.6±3.4% *versus* 45.0±2.7%, p<0.0001, two-tailed paired Student's *t*-test). Post-partum levels were lower than in the non-pregnant women (45.0±2.7% *versus* 57.7±2.8%, p=0.006, two-tailed unpaired Student's *t*-test). Monocyte CD40 expression was not significantly different in pre-eclampsia. Although the post-partum data is combined graphically for the women with pre-eclampsia, there was no difference in levels of expression between women with pre-term or term pre-eclampsia at the post-partum visit (two-tailed unpaired Student's *t*-test; p=0.9, Figure 28).

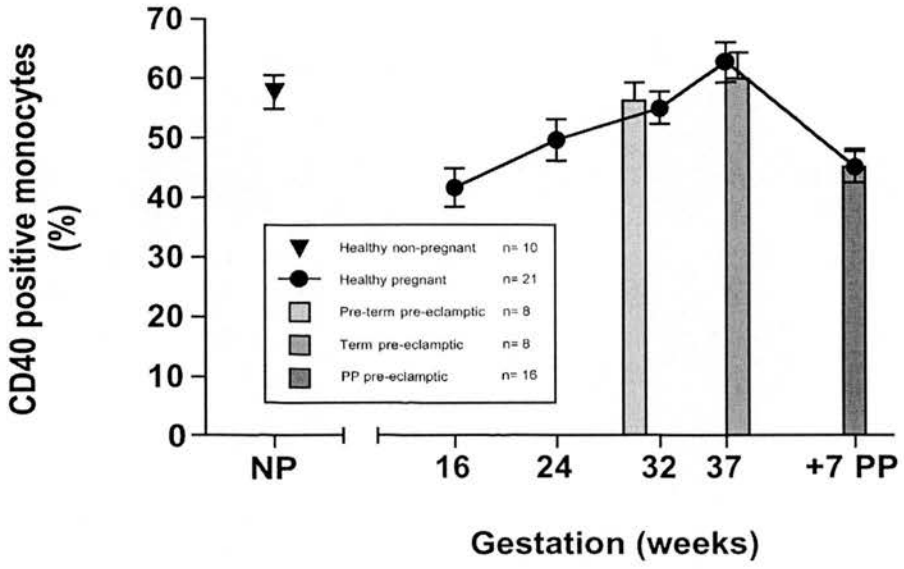

Figure 28. Influence of normal and pre-eclamptic pregnancy on monocyte CD40 expression Monocyte CD40 expression was lower at 16 weeks than in non-pregnant women (p=0.0009, two-tailed unpaired Student's *t*-test) varied with gestation (p<0.0001, one-way ANOVA with repeated measures), rising between 16 and 32, 16 and 37, 24 and 37 weeks (p<0.01 for all, Bonferroni's post-test) and decreased post-partum (p<0.0001; 37 weeks *versus* post-partum, two-tailed paired Student's *t*-test). CD40 expression was not significantly different in pre-eclampsia. Data expressed as mean±SEM.
NP - non-pregnant; PP - post-partum; CD - cluster of differentiation; SEM - standard error of the mean.

In contrast, monocyte CD11b expression was greater in healthy pregnant women, at 16, 24 and 37 weeks than in non-pregnant women (69.1±7.5, 57.2±4.6 and 59.6±4.9 *versus* 43.9±3.3 MFI, p<0.02 for all, two-tailed unpaired Student's *t*-tests), though it did not vary significantly with gestation (p=0.06, one-way ANOVA with repeated measures). Levels of expression fell post-partum (37 weeks *versus* post-partum, 59.6±4.9 *versus* 47.9±3.3 MFI, p=0.02; two-tailed paired Student's *t*-test), returning to non-pregnant levels. CD11b expression was greater in women with pre-term pre-

eclampsia than women with healthy pregnancies at 32 weeks gestation (73.8±7.2 *versus* 50.8±3.0 MFI, p=0.001, two-tailed unpaired Student's *t*-test), but not significantly different in women with term pre-eclampsia, or post-partum compared to healthy pregnant women. Although the post-partum data is combined graphically for the women with pre-eclampsia, there was no difference in CD11b expression between women with pre-term or term pre-eclampsia at the post-partum visit (two-tailed unpaired Student's *t*-test; p=0.4, Figure 29).

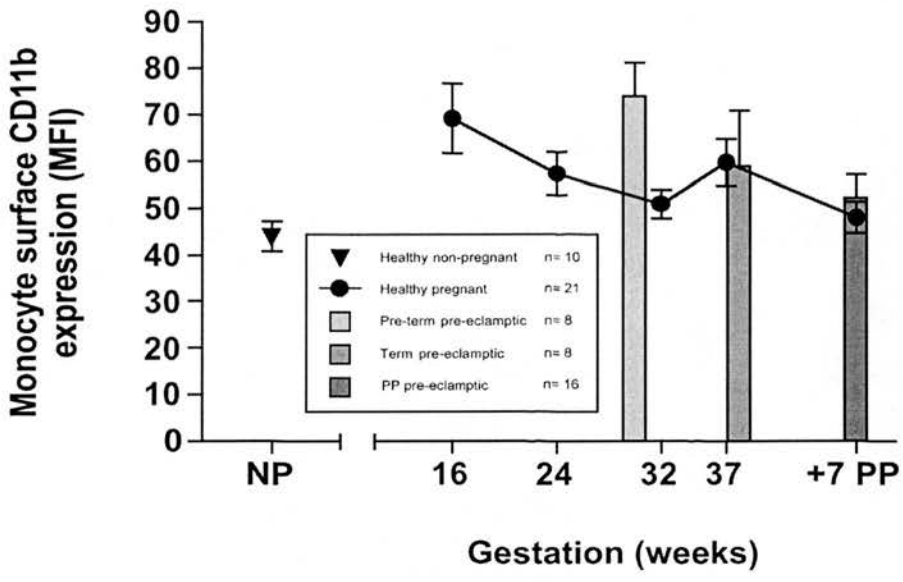

Figure 29. Influence of normal and pre-eclamptic pregnancy on monocyte CD11b expression. Monocyte CD11b expression was greater at 16, 24 and 37 weeks gestation than in non-pregnant women (p<0.02 for all, two-tailed unpaired Student's *t*-tests). It did not vary significantly with gestation (p=0.06, one-way ANOVA with repeated measures), but fell post-partum (p=0.02; 37 weeks *versus* post-partum, two-tailed paired Student's *t*-test). CD11b expression was greater in pre-term pre-eclamptic pregnancies than healthy pregnancy at 32 weeks gestation (p=0.001, two-tailed unpaired Student's *t*-test), but not significantly different in term pre-eclampsia, or post-partum. Data expressed as mean±SEM.

NP - non-pregnant; PP - post-partum; CD - cluster of differentiation; MFI - mean fluorescent intensity; SEM - standard error of the mean.

4.4 DISCUSSION

This longitudinal study has demonstrated that platelet-monocyte aggregates, in conjunction with other markers of platelet activation, are increased in the third trimester of pregnancy compared to the non-pregnant state. In addition, it has demonstrated that both surface expression of platelet P-selectin and soluble plasma concentration increase with gestation. However, none of the markers of platelet activation were affected by the menstrual cycle or pre-eclampsia. A novel finding of this study was that plasma soluble CD40L did not increase in a similar manner to the other markers of platelet activation, but paradoxically fell during normal pregnancy between 16 and 24 weeks. In a similar pattern monocyte CD40 expression was also decreased at 16 weeks gestation. CD11b was elevated in healthy pregnancy, reflecting increased monocyte activation, and levels were higher in samples from women with pre-term pre-eclampsia. This leads to the conclusion that both platelet and monocyte activation contribute to the prothrombotic state during pregnancy but that systemic platelet activation is not a feature of established pre-eclampsia.

4.4.1 PLATELET AND MONOCYTE ACTIVATION WITHIN THE MENSTRUAL CYCLE

Given that during the menstrual and early follicular phase of the cycle, the priority is to secure haemostasis and prevent excessive blood loss, it was anticipated that an increase in platelet activation would be detected at this time. However, in the current study there was no variation in platelet or monocyte activation during the menstrual cycle. These findings appear to differ from the only previous study on platelet-leukocyte activation during the menstrual cycle by Rosin *et al* [2006]. They found that platelet-leukocyte aggregates peaked mid-cycle, corresponding to ovulation

[Rosin *et al*, 2006]. A variety of factors may account for these seemingly discrepant findings, but in particular, the differing methodologies employed between their study and this one. Rosin *et al* [2006] used a small gauge butterfly needle for venesection, collected the blood into citrated tubes for flow cytometry and undertook initial processing within 20 minutes of sampling. It has been recently demonstrated that all these factors have a marked effect on platelet-monocyte aggregation [Harding *et al*, 2007]. The formation of platelet-monocyte aggregates is calcium dependent and will therefore be inhibited by anticoagulation with citrate. Moreover, the small gauge needle used by Rosin *et al* [2006] may have caused artifactual platelet activation, particularly if there was a long connecting tube between the needle and citrated collecting tube. Finally, platelet-monocyte aggregates increase in a time-dependent manner *in vitro.* Rosin and colleagues [Rosin *et al*, 2006] may not have processed their samples until 20 minutes after original venepuncture. In contrast, this current study used a large needle for venesection, employed a direct thrombin inhibitor as an anticoagulant, and immunolabelled all samples at exactly 5 minutes after collection.

This is the first study to examine surface expression of monocyte CD40 and CD11b in the menstrual cycle. Previous studies addressing monocyte activation within the menstrual cycle have focused on measuring plasma concentrations, or basal and stimulated cytokine and chemokine release from monocytes [Angstwurm *et al*, 1997; Brannstrom *et al*, 1999; Willis *et al*, 2003; Timmons *et al*, 2005]. The results are conflicting, with greater cytokine and chemokine release (IL-6, TNF-a) from monocytes in the luteal versus follicular phase [Willis *et al*, 2003] (stimulated TNF-a), and follicular versus luteal phase [Angstwurm *et al*, 1997] (plasma IL-6) [Brannstrom *et al*, 1999] (plasma TNF-a), or no change [Timmons *et al*, 2005]

(plasma IL-6). However, plasma concentrations of cytokines and chemokines are an indirect method of assessing moncyte activation, as they may also be released from other cells, for example, endothelial cells release IL-6. Assessing monocyte surface expression of molecules, in whole blood, using flow cytometry provides a more sensitive assessment of *in vivo* monocyte activation status. Although monocytes are known to be activated by both oestradiol and progesterone, and express oestrogen receptors, [Li *et al*, 1993; Ben-Hur *et al*, 1995] in this longitudinal study no clear effect of cycling ovarian hormones on CD40 or CD11b expression was found.

4.4.2 Platelet and monocyte activation within healthy pregnancy

Pregnancy is associated with a hypercoagulable state with an increased risk of both venous and arterial thromboembolism [Simpson *et al*, 2001]. However the contribution that platelets make to this pro-inflammatory, thrombotic state is less clear with some studies reporting increased activation [Gerbasi *et al*, 1990; Janes and Goodall, 1994] while others reporting no change [Romero *et al*, 1988; Gatti *et al*, 1994; Star *et al*, 1997]. Using both cellular and soluble markers of platelet activation this study demonstrates a gestational increase in platelet activation compared to the non-pregnant state. This longitudinal study adds to and supports the recent cross-sectional study of Holthe and colleagues [Holthe *et al*, 2005] in which they demonstrate an increase in platelet-leukocyte aggregates at a single time point during the third trimester compared to matched controls sampled at a single unspecified time-point during the menstrual cycle.

Previous cross-sectional studies in pregnancy have used a variety of different techniques including platelet aggregation [Yamazaki *et al*, 1979; Markham *et al*,

1991; Holthe *et al*, 2005], platelet fibrinogen activation [Faraday *et al*, 1997; Heilmann *et al*, 2007], P-selectin expression [Karalis *et al*, 2005, Borzychowski *et al*, 2006] and platelet count [Larsen *et al*, 1996]. By using a complementary panel of cellular and circulating markers of platelet activation, the current study suggests that pregnancy is a state of continued *in vivo* platelet activation. In addition, the longitudinal study design provides important insights into platelet activation in varying hormonal states in pregnant and non-pregnant women.

Consistent with the finding of increased platelet activation in pregnancy, there is increased expression of monocyte CD11b in healthy pregnancy. Activated platelets, binding to monocytes up-regulate CD11b expression and promote inflammatory cytokine release. These findings are consistent with previously published data, which demonstrate an increase in CD11b in the third trimester of pregnancy [Sacks *et al*, 1998] but no change with gestation [Luppi *et al*, 2002]. It has been proposed that increased systemic monocyte activation may be involved in the initiation of labour, [Luppi *et al*, 2004]. However, the findings from this study suggest that activation occurs earlier in gestation, though trafficking of these monocytes may be altered near term.

4.4.3 PLATELET AND MONOCYTE ACTIVATION AND PRE-ECLAMPSIA

Pre-eclampsia is associated with systemic endothelial dysfunction and often local thrombotic changes within the placenta. Although it is often assumed that the inflammatory milieu of pre-eclampsia is accompanied by platelet activation, the existing literature is controversial with studies demonstrating that platelet activation may be increased [Konijnenberg *et al*, 1997; Harlow *et al*, 2002; Bagamery *et al*,

2005] or unchanged [Holthe *et al*, 2005; Acar *et al*, 2007; Lok *et al*, 2007] compared to normal pregnancy. It was not possible to demonstrate a difference in platelet activation in any of the measures in this study between the subjects with pre-eclampsia and healthy pregnant women. These findings are consistent with the cross-sectional study by Holthe *et al* [2005] which reported no difference in platelet-leukocyte aggregates between normal pregnancy and pre-eclampsia [Holthe *et al*, 2005], but do not support their earlier study of increased basal P-selectin expression on platelets from women with pre-eclampsia [Holthe *et al*, 2004a]. The finding that plasma P-selectin concentrations are unchanged, support those of Lok *et al* [2007]. The present study is limited by the lack of data for women prior to the development of pre-eclampsia. The increased capacity for platelets to aggregate following *in-vitro* stimulation is more pronounced at earlier gestations, in women destined to develop pre-eclampsia, with the difference reducing during the third trimester [Felfernig-Boehm *et al*, 2000]. These results do not exclude a role for platelet activation prior to the development of pre-eclampsia or the presence of local platelet activation at the foeto-maternal interface, but we could not find evidence of increased systemic platelet activation in established pre-eclampsia above that caused by pregnancy.

This research demonstrates that pre-term pre-eclampsia is associated with an increase in monocyte CD11b expression compared to healthy normotensive pregnant women at similar gestations. This finding is supportive of previously published work that demonstrates increased CD11b expression [Holthe *et al,* 2004b], cytokine and chemokine release form the monocytes of pre-eclamptic women [Luppi and Deloia, 2006]. Interestingly, no associated platelet activation was demonstrated, suggesting involvement of other stimulatory pathways. In addition, this up-regulation was not

seen in the term-pre-eclamptic women, which may reflect a difference in the pathogenesis of pre-eclampsia in these women.

4.4.4 CD40-CD40 LIGAND DYAD IN PREGNANCY

A novel finding of this study is that plasma soluble CD40L concentrations were lower in pregnant women at mid gestation, falling from 16 to 24 weeks and then gradually rising during the third trimester. This pattern was also reflected in monocyte CD40 expression. There was no difference in either soluble CD40L concentration or monocyte CD40 expression in women in the third trimester (both with and without pre-eclampsia) compared to non-pregnant women. However, soluble CD40L concentrations increased and monocyte CD40 expression decreased, post-partum in both groups of women. This gestational variation in soluble CD40L concentrations and monocyte CD40 expression has not been reported previously. As soluble CD40L is predominately released by activated platelets, an increase was expected as other markers of platelet activation were elevated. This lack of correlation between soluble CD40L and platelet bound or soluble P-selectin might reflect different aspects of platelet biology and has reported in patients outwith pregnancy [Blann *et al*, 2005].

Our study adds to the findings of a previous cross-sectional study, which demonstrated that soluble CD40L concentrations were lower during pregnancy compared to the non-pregnant state [Oron *et al*, 2006]. However, unlike this current study, Oron and Mellembakken reported increased soluble CD40L concentrations in pre-eclampsia [Mellembakken *et al*, 2001; Oron *et al* 2006]. This again is most probably due to methodological differences. Pre-analytical conditions, sampling technique and storage have a clear effect on soluble CD40L concentrations and are

not yet standardised for this marker. Oron *et al* [2006], measured soluble CD40L concentrations in serum, which are higher than in plasma, less stable and more vulnerable to sample processing [Mason *et al*, 2005; Weber *et al*, 2006]. Although Mellembakken *et al* [2006] measured plasma soluble CD40L, this was following preparation and centrifugation of platelet rich plasma. The study presented in this chapter, measured plasma soluble CD40L with minimal processing and risk of *ex vivo* platelet activation.

No studies have examined the effect of monocyte CD40 expression in pregnancy. A limitation of our study is that there is no available data on the surface expression of CD40L on platelets. Following previously published flow cytometric techniques, surface CD40L expression was studied but we were unable to find a monoclonal antibody that detected specific immunoreactivity above a matched isotype control. However, over 95% of circulating soluble CD40L is thought to be derived form platelets and its function is reported to be preserved between the membrane bound and soluble form [Danese and Fiocchi, 2005; Li *et al*, 2008].

Unlike direct markers of platelet and monocyte activation, the CD40-CD40 ligand dyad is more complex and has other pro-inflammatory functions. It is expressed on a wide variety of immune cells, is involved in humoral and cellular immunity through lymphocyte activation and differentiation, and thymic selection. There is extensive research to support the central role of the soluble biologically active CD40L molecule in immune responses, the development of autoimmunity and in inflammatory disorders [van Kooten, 2000]. Given the paradoxical suppression of CD40L and monocyte CD40 compared to the other markers of platelet activation and monocyte

CD11b, it is likely that the CD40-CD40 ligand dyad may have other, perhaps immunomodulatory roles, such as at the materno-foetal interface that are not yet fully characterised in pregnancy.

4.5 SUMMARY

In summary, this chapter has demonstrated using sensitive flow cytometric techniques that normal pregnancy is associated with platelet and monocyte activation that increases with gestation. In addition, systemic platelet activation does not appear to be a feature of the menstrual cycle or established pre-eclampsia. Monocyte activation does appear to be associated with pre-term pre-eclampsia but is unaltered by the varying hormonal milieu of the menstrual cycle. A novel variation in the CD40-CD40 ligand dyad in pregnancy is also described.

Although the requirement to process samples immediately for flow cytometric studies poses logistical challenges, future areas of research would focus on both platelet and monocyte activation in the first trimester of pregnancy and ideally in women prior to the development of pre-eclampsia.

CHAPTER 5

THE INFLUENCE OF THE MENSTRUAL CYCLE, PREGNANCY AND PRE-ECLAMPSIA ON CIRCULATING ENDOTHELIAL PROGENITOR CELLS

Robb AO, Mills NL, Smith IBJ, Short A, Tura-Ceide O, Barclay GR, Blomberg A, Critchley HOD, Newby DE, Denison FC
Influence of menstrual cycle on circulating endothelial progenitor cells.
Human Reproduction 2009;**24** (3):619-625.

5.1 INTRODUCTION

Endothelial progenitor cells are circulating mononuclear cells derived from the bone-marrow, which have the potential to proliferate, migrate and differentiate into mature endothelial cells. Increasingly they are recognised as having an important role in mediating vascular endothelial repair and angiogenesis [Urbich and Dimmeler, 2004]. Their mobilisation and function is affected by inflammation, hormonal regulation and endothelial dysfunction. Indeed, these three factors, as well as endothelial repair, are pivotal to many reproductive processes including menstruation, healthy pregnancy and complications of pregnancy such as pre-eclampsia.

Currently there is a lack of consensus on the definitive EPC phenotype with a variety of surface markers and functional assays being used to assess this progenitor population. This makes comparison between different clinical studies difficult. Although groups are increasingly quantifying EPCs by surface expression of phenotypic markers ($CD34^+CD133^+KDR^+$), the CFU-EPC assay represents a functional assessment that quantifies the ability of putative endothelial progenitors to form colonies [Hill *et al*, 2003]. To date relatively few clinical studies have either used both approaches or compared EPC phenotype and function.

The aim of this research was to determine the influence of the menstrual cycle, normal and pre-eclamptic pregnancy on the number and function of circulating EPCs, and to investigate how their number and function vary with cyclical variation of circulating sex steroids or inflammatory mediators. We hypothesised that EPCs

are mobilised during inflammatory reproductive processes involving endothelial repair. In this chapter we address the following hypotheses:

(i) Circulating EPC numbers and function (assessed by flow cytometry and CFU-EPC assay) would vary during the menstrual cycle, healthy pregnancy and in pregnancies complicated by pre-eclampsia.

(ii) Circulating EPC numbers and function would correlate with serum hormone levels (oestradiol and progesterone) and measures of inflammation, IL-6, TNF-α, soluble ICAM-1 and VEGF.

It was anticipated that the results of these investigations could contribute to our understanding of the role of EPCs in common reproductive events.

5.2 METHODS

All materials, reagents and cell lines used are detailed in Appendix 1.

5.2.1 SUBJECT RECRUITMENT

Women were identified, recruited and consented to this study (study 2, Reference number 05/S1104/48), as described in section 2.1.

Non-pregnant women

Healthy pre-menopausal non-smoking nulliparous women (n=16) with at least a 2-month history of normal regular menstrual cycles were recruited to the study. Exclusion criteria are detailed in section 2.1.2.

Pregnant women

Healthy, non-smoking primigravida women with an uncomplicated pregnancy (n=30) or with pre-eclampsia (n=16) were recruited in the first trimester of pregnancy or at diagnosis respectively. Women were given a minimum of 24 hours to consider participating in the study. Exclusion criteria are detailed in section 2.1.3.

5.2.2 VISIT SCHEDULE

Women abstained from alcohol, caffeine and tobacco for 12 hours and fasted for 4 hours prior to attendance. Blood pressure and heart rate were recorded in duplicate using an automated sphygmomanometer following a 15 minute left-lateral or supine rest for pregnant subjects (at every visit) and non-pregnant (at first visit, D 1-3) respectively. Height and weight were also measured to allow calculation of BMI. Women with pre-eclampsia continued to take their antihypertensive medication as prescribed. This is detailed in section 5.3.1.

Non-pregnant women

Women attended for four visits during a single menstrual cycle (D 1-3 (early follicular), D 6-8 (mid-follicular), D 13-15 (peri-ovulatory) and D 20-22 (mid-luteal)).

Pregnant women

Women with uncomplicated pregnancies attended for four visits during pregnancy at 16, 24, 32 and 37 weeks and at 7 weeks post-partum. Women with pre-eclampsia attended following diagnosis and at 7 weeks post-partum.

5.2.3 Sample collection

At each visit, peripheral venous blood was drawn from a large antecubital vein as described in section 2.4.2. Venous blood collected in tubes containing EDTA was used for the CFU-EPC assay (10 mL) and for flow cytometry (2 mL). The full blood count differential was obtained from a 5 mL sample of EDTA-anticoagulated blood processed by the hospital haematology laboratory. Plasma and serum were prepared and stored as described in section 2.4.2 for subsequent analysis of circulating hormones and inflammatory mediators. Following venepuncture, arterial stiffness studies were performed, detailed in Chapter 6.

5.2.4 In vitro culture

Functional EPCs were quantified using the Endocult™CFU-EPC assay as described in section 2.6.1.

5.2.5 Immunofluorescence

This was performed to confirm endothelial cell lineage of the colonies formed within the CFU-EPC assay and is described in section 2.7.1.

5.2.6 Flow cytometric analysis of EPCs

This was performed as described in section 2.5.4.

5.2.7 Measurement of circulating hormones

Serum concentrations of oestradiol and progesterone were measured in all women. Lutenizing hormone and FSH were also measured in the healthy non-pregnant women. All hormones were measured by automated chemiluminescent microparticle assays as described in section 2.9.1-4.

5.2.8 Measurement of inflammatory markers

ELISAs were used to measure plasma concentrations of TNF-α, IL-6 and soluble ICAM-1 in all women, as described in section 2.8.3-5. In addition, plasma VEGF was measured by ELISA in the healthy non-pregnant women, as described in section 2.8.6.

5.2.9 Statistical analysis

The distribution of data was determined using the Kolmogorov-Smirnov test for normality. Continuous variables are reported as mean±SEM, or median±interquartile range for parametric and non-parametric data respectively. Statistical analyses were performed using two-tailed Student's *t*-tests or Mann-Whitney tests for parametric and non-parametric data respectively. For multiple comparisons, one-way ANOVA with repeated measures and Bonferroni's post-tests or non-parametric Friedman analyses were used. Correlation coefficients were calculated using Pearson or

Spearman analyses for parametric and non-parametric data respectively. All calculations were performed using GraphPad Prism, (GraphPad Software, USA). Statistical significance was taken at 5%.

5.3 RESULTS

5.3.1 SUBJECT CHARACTERISTICS

Non-pregnant women

In six of sixteen non-pregnant women, ovulation could not be confirmed leaving a final study group of ten women with a confirmed regular ovulatory cycle (cycle length 28±0.4 days; luteal phase serum progesterone >30 nmol/L). All women (age, 31.4±2.0 yr) were of normal body composition (BMI, 23.4±0.7 kg/m^2). Baseline systolic and diastolic blood pressures and heart rate, measured at D 1-3 of the cycle, were 110±2 mmHg, 67±2 mmHg and 60±2 bpm, respectively.

Pregnant women

In the healthy pregnant group, four of thirty women developed complications in their pregnancy, for example intra-uterine growth restriction, pre-term labour or pre-eclampsia and a further five were unable to complete the full schedule of visits. In addition technical problems with the flow cytometer resulted in samples from two women being unanalysed. Overall, the final study group consisted of complete samples from nineteen women. In the pre-eclamptic group, women were categorised according to gestation at presentation, into pre-term (n=8; mean 30 (range 24-34

weeks)) and term (n=8; mean 38 (range 36-40 weeks)). Women with pre-eclampsia were delivered earlier than women with uncomplicated pregnancies and had offspring of lower birth weight (Table 5).

TABLE 5. BASELINE CHARACTERISTICS OF STUDY PARTICIPANTS

	Healthy non-pregnant group (n=10)	Healthy pregnant group (n=19)	Pre-eclamptic group (n=16)	
			Pre-term (n=8)	Term (n=8)
Age (years)	31±2	30±1	28±3	30±2
Height (m)	1.70±0.02	1.65±0.02*	1.63±0.02*	1.62±0.02*
Weight (Kg)	68±3	72±4	64±4	75±4
Body Mass Index (kg/m2)	23±1	27±1	24±2	29±1*
Gestation at delivery (weeks)	N/A	41±0.3	31±1.4 ‡	39±0.4†
Birth weight (g)	N/A	3430±120	1366±192§	3231±252

Data expressed as mean±SEM. Body mass index was recorded during first trimester for pregnant women.
$p<0.05$ *versus* healthy non-pregnant group, † $p\leq0.0009$ *versus* healthy non-pregnant group, ‡ $p\leq0.0003$ *versus* healthy pregnant group,
§ $p<0.0001$ *versus* healthy pregnant and term pre-eclamptic groups, (two-tailed unpaired Student's *t*-tests).
SEM - standard error of the mean.

There was no difference in first trimester blood pressures between women with healthy and pre-eclamptic pregnancies (113±2/69±2 mmHg *versus* 117±3/73±2 mmHg, $p>0.9$). As expected women, with pre-term and term pre-eclampsia had higher blood pressures at the time of study compared to those women with uncomplicated pregnancies at similar gestations (Pre-term pre-eclampsia, (30 weeks) *versus* healthy pregnancy, (32 weeks) 140±2/87±3 mmHg *versus* 112±1/70±1 mmHg respectively; term pre-eclampsia (38 weeks) *versus* healthy pregnancy (37 weeks) 147±2/96±2 mmHg *versus* 117±2/76±1 mmHg respectively; all $p<0.0001$). The women with pre-eclampsia were no longer hypertensive at the post-partum visit, but still had higher blood pressures than the women with uncomplicated pregnancies (pre-eclampsia *versus* healthy pregnancy post-partum; 123±3/77±2 mmHg *versus* 113±1/69±1 mmHg, both $p<0.002$).

Medication use

In women with pre-term pre-eclampsia, five were taking regular labetalol and nifedipine, two were taking regular methyldopa and nifedipine, and one was receiving no anti-hypertensive therapy. Seven of these women received antenatal betamethasone. In women with term pre-eclampsia, one was taking regular labetalol with the remaining seven women not receiving anti-hypertensive therapy. None received antenatal betamethasone. Post-partum, out of the original sixteen women who had developed pre-eclampsia, only three women were taking labetalol, and one, methyldopa.

5.3.2 THE CONCENTRATION OF CIRCULATING TRIPLE POSITIVE $CD34^+CD133^+KDR^+$ EPCS VARIED THROUGHOUT THE MENSTRUAL CYCLE

Concentrations of circulating triple positive $CD34^+CD133^+KDR^+$ EPCs varied throughout the menstrual cycle ($p=0.04$, one-way ANOVA with repeated measures) with mid-follicular levels ($0.99\pm0.3 \times 10^6$ cells L^{-1}) being 3-fold higher than peri-ovulatory levels ($0.29\pm0.1 \times 10^6$ cells L^{-1}, $p<0.05$, Bonferroni's post-test for multiple comparisons; Figure 30).

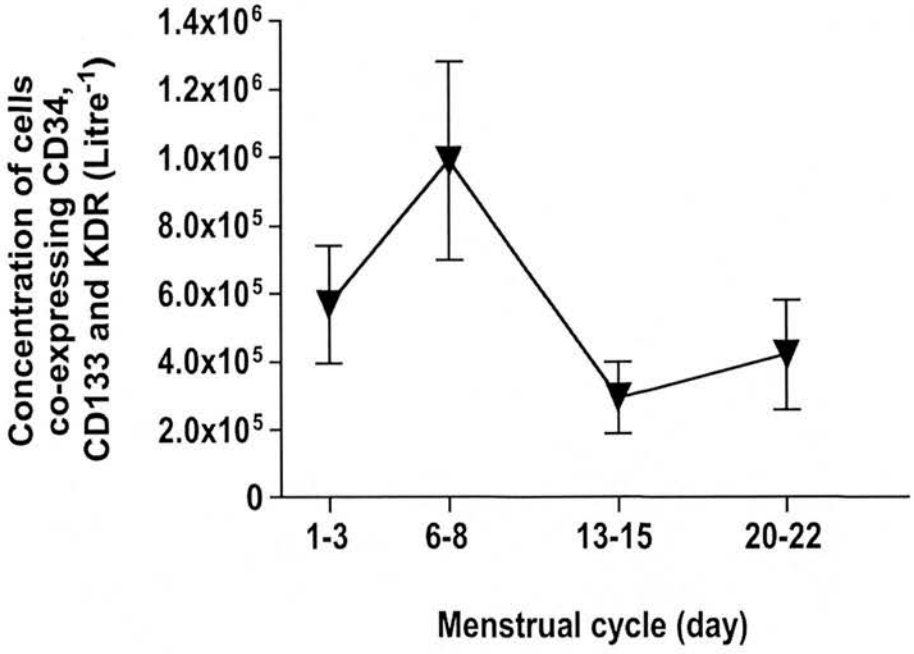

Figure 30. Concentration (mean±SEM) of leucocytes co-expressing CD34, CD133 and KDR varied throughout the menstrual cycle (n=10, p=0.04, one-way ANOVA with repeated measures). Mid-follicular levels were higher than peri-ovulatory levels ($p<0.05$, Bonferroni's post-test for multiple comparisons).
SEM - standard error of the mean; CD - cluster of differentiation; KDR - kinase insert domain receptor; ANOVA - anlaysis of variance.

5.3.3 The number of CFU-EPCs formed did not vary throughout the menstrual cycle

In contrast, there was no variation in numbers of CFU-EPCs formed during the menstrual cycle (p=0.6; Figure 31). Direct staining confirmed that CFU-EPCs, like mature endothelial cells, bind lectin, integrate AcLDL and are positive for the endothelial markers CD105 and CD146 (Chapter 2, Figure 14). There was no correlation between EPCs quantified by phenotype ($CD34^+CD133^+KDR^+$) and number of functional CFU-EPCs during the menstrual cycle.

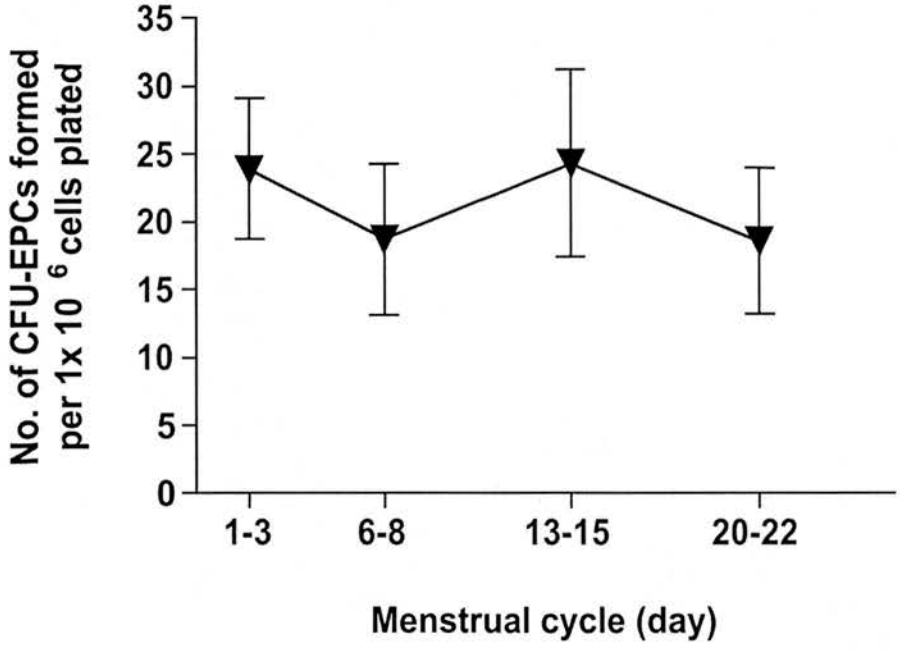

Figure 31. Number (mean±SEM) of CFU-EPCs formed per 1 x 10^6 mononuclear cells plated, throughout the menstrual cycle. (n=10, p=0.6, one-way ANOVA with repeated measures).
SEM - standard error of the mean; CFU-EPCs - colony forming unit-endothelial progenitor cells; ANOVA - anlaysis of variance.

5.3.4 SEX STEROIDS AND INFLAMMATORY MEDIATORS

There was normal cyclical variation in circulating pituitary and ovarian hormones with peri-ovulatory peaks in serum LH and FSH concentrations, and peri-ovulatory and mid-luteal peaks in serum oestradiol and progesterone concentrations respectively (Table 6). Concentrations of plasma TNF-α ($p=0.05$) but not IL-6, soluble ICAM-1 or VEGF ($p=0.51$, $p=0.36$, $p=0.84$, respectively) varied over the menstrual cycle. There was no relationship between functional or phenotypic EPCs and circulating levels of oestradiol, progesterone, soluble ICAM-1, IL-6 or VEGF at any phase of the menstrual cycle. A weak correlation was seen between plasma levels of TNF-α and concentration of phenotypic EPCs ($r=0.33$, $p=0.04$).

TABLE 6. HORMONAL AND INFLAMMATORY PROFILE OF NON-PREGNANT WOMEN

	Day of menstrual cycle				
	1-3	**6-8**	**13-15**	**20-22**	**Significance**
Day at sampling	2±0.2	7±0.2	14±0.3	21±0.2	
LH (UL^{-1})	5±0.4	7±0.4	32±7.2	5±0.5	p<0.0001
FSH (UL^{-1})	6±0.4	6±0.4	9±1.9	3±0.2	p=0.002
Oestradiol (pmol L^{-1})	180±21.1	318±68.4	1342±274.4	827±93.4	p<0.0001
Progesterone (nmol L^{-1})	4±0.3	3±0.2	7±2.1	47±4.5	p<0.0001
SICAM-1 ($ngmL^{-1}$)	214±15.2	193±11.6	203±8.1	199±11.8	p=0.36
TNF-α ($pgmL^{-1}$)*	0.73 (0-3)	0.41 (0-0.9)	0.075 (0-1)	0.0 (0-0.04)	p=0.05
IL-6 ($pgmL^{-1}$)*	0.13 (0.1-0.2)	0.11 (0.1-0.2)	0.095 (0.06-1)	0.14 (0.07-0.5)	p=0.51
VEGF ($pgmL^{-1}$)*	32.9 (1-72.9)	32.1 (1-89.7)	39.5 (1-56.4)	43.4 (1-58.5)	p=0.84

Data expressed as mean±SEM or *median with inter-quartile ranges. Circulating LH, FSH, oestradiol and progesterone varied significantly throughout the menstrual cycle. Plasma TNF-α concentration decreased, whilst levels of IL-6, sICAM-1 and VEGF did not vary significantly.
LH - lutenising hormone; FSH - follicle stimulating hormone; TNF-α - tumour necrosis factor-α; IL - interleukin;
sICAM - soluble intercellular adhesion molelcule-1; VEGF - vascular endothelial growth factor; SEM - standard error of the mean.

5.3.5 THE CONCENTRATION OF CIRCULATING TRIPLE POSITIVE $CD34^+CD133^+KDR^+$ EPCS DID NOT VARY SIGNIFICANTLY IN NORMAL PREGNANCY AND WAS NOT AFFECTED BY PRE-ECLAMPSIA

There was no significant variation in concentrations of leucocytes co-expressing CD34, CD133 and KDR with gestation in healthy pregnancy (p=0.6; Figure 32). Although numbers appear to be higher in the pre-term pre-eclamptic women, this was not significant and was due to their increased total leucocyte concentration, ($1.3x10^{10}$ ±1.5 x 10^9 *versus* 9.3±4.2 x 10^9 L^{-1}, p=0.005, two-tailed, unpaired Student's *t*-test). Six of these women had received antenatal betamethasone, which increases the granulocyte fraction of the circulating white count [Kadanali *et al*, 1997]. No healthy pregnant women or women with pre-eclampsia at term received betamethasone.

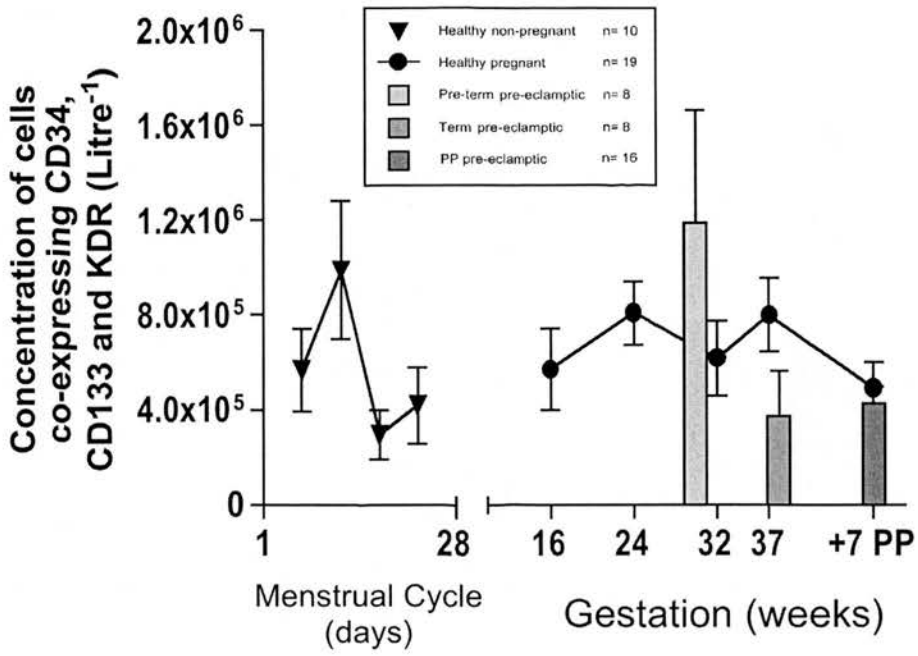

Figure 32. Concentration (mean±SEM) of leucocytes co-expressing CD34, CD133 and KDR in healthy non-pregnant, pregnant and pre-eclamptic women. No difference was seen within healthy pregnancy (p=0.6, one-way ANOVA with repeated measures). Apart from the variation within the menstrual cycle (described earlier in Figure 30), no other differences were observed between healthy non-pregnant women, healthy pregnant and pre-eclamptic women. There was no difference in concentration between women with pre-term or term pre-eclampsia at the post-partum visit (two-tailed unpaired Student's *t*-test; p=0.3)
SEM - standard error of the mean; CD - cluster of differentiation; KDR - kinase insert domain receptor; ANOVA - analysis of variance; PP - post-partum..

5.3.6 The number of CFU-EPCs formed in normal pregnancy and in pre-eclampsia varied widely

The numbers of CFU-EPCs formed from mononuclear cells from pregnant women varied widely. There was no difference in numbers of CFU-EPCs formed throughout pregnancy (p=0.42, Friedman test; Figure 33). In addition, numbers formed were not different between healthy pregnant women and those with pre-eclampsia (p>0.1 for all, Mann-Whitney test). There was no relationship between functional or phenotypic EPCs in healthy pregnancy or pre-eclampsia.

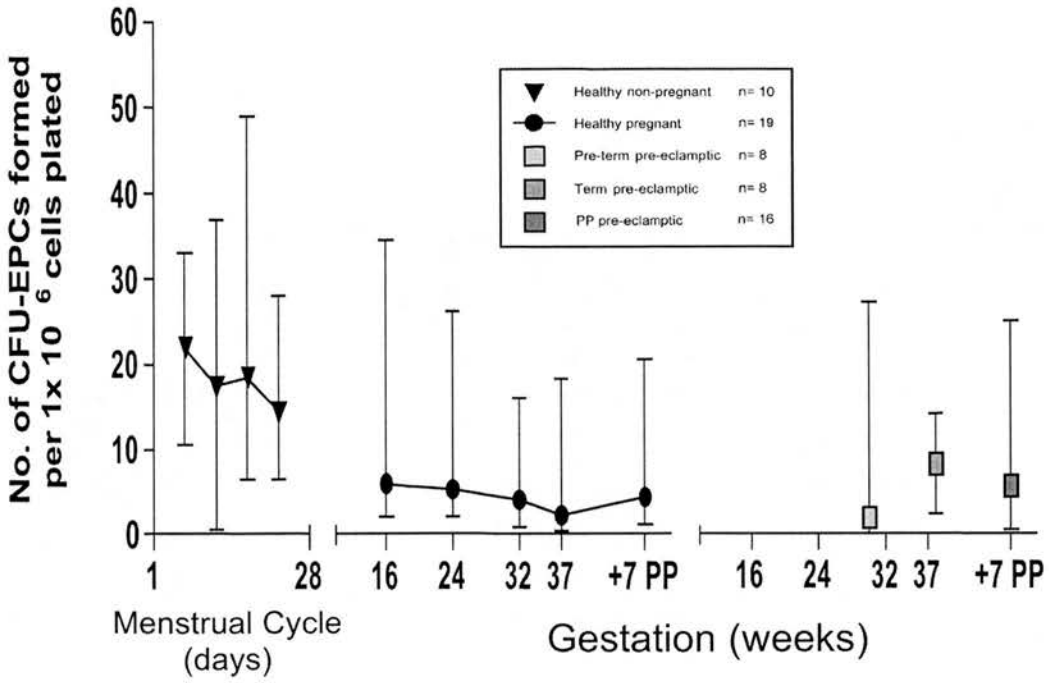

Figure 33. Number (median±interquartile range) of CFU-EPCs formed per 1 x 10^6 mononuclear cells plated, throughout the menstrual cycle, healthy and pre-eclamptic pregnancies. Data for menstrual cycle is expressed as median±interquartile range to allow for comparison between groups. There was no significant variation within pregnancy ($p=0.42$, Friedman test) or between the healthy pregnant and pre-eclamptic groups ($p>0.1$ for all, Mann-Whitney test). There was no difference in numbers formed between women with pre-term or term pre-eclampsia at the post-partum visit (Mann-Whitney test; $p>0.05$). Numbers of CFU-EPCs were lower in pregnancy, from 24 weeks and post-partum (both groups), than in the early follicular phase of the menstrual cycle ($p<0.04$ for all, Mann-Whitney test). CFU-EPCs - colony forming units-endothelial progenitor cells; PP - post-partum.

The number of CFU-EPCs formed was lower in healthy pregnancy (from 24 weeks) and post-partum (healthy pregnant and pre-eclamptic groups), than the early follicular phase of the menstrual cycle ($p<0.04$ for all, Mann-Whitney test). Log transformation of the data reveals similar results with a lower number of CFU-EPCs formed in the third trimester of healthy pregnancy than in the early follicular phase of the menstrual cycle ($p<0.02$, Mann-Whitney test). We observed a greater proportion of samples from healthy pregnant women to be poorly proliferative compared to samples from healthy non-pregnant women. Fifty-one per cent of all

samples taken in pregnancy from healthy pregnant women grew five colonies or fewer compared to 15% of samples from the healthy non-pregnant women, from throughout the cycle ($p<0.05$, Chi-squared test).

5.3.7 THERE WAS NO CONSISTENT CORRELATION BETWEEN TRIPLE POSITIVE $CD34^+CD133^+KDR^+$ EPCS, CFU-EPCS AND CIRCULATING SEX STEROIDS OR INFLAMMATORY MEDIATORS

Serum oestradiol and progesterone

Expected increases with gestation were observed in pregnant women in serum oestradiol and progesterone concentrations (Table 7). Women with pre-term pre-eclampsia had lower serum oestradiol levels than healthy pregnant women at 32 weeks gestation (29950±9876 *versus* 50297±2683 pmol L^{-1}, $p=0.009$, two-tailed unpaired Student's *t*-test). There was no relationship between phenotypic EPCs and circulating levels of oestradiol or progesterone. A positive correlation was seen between number of CFU-EPCs and oestradiol ($r=0.48$, $p=0.04$) and progesterone ($r=0.6$, $p=0.0093$) in samples from the 24-week visit only.

Plasma TNF-α

Concentrations of plasma TNF-α were not different in pregnancy compared to the non-pregnant state (at any phase of the cycle) or the post-partum visit. Concentrations of plasma TNF-α did not vary significantly within pregnancy, nor were they different in the women with pre-eclampsia compared to the healthy pregnant women ($p>0.2$ for all, Freidman and Mann-Whitney tests; Table 7).

A negative correlation was seen between plasma levels of TNF-α and concentration of phenotypic EPCs in samples obtained from women at 16 and 24 weeks gestation ($r=-0.47$, $p=0.04$ and $r=-0.5$, $p=0.03$, Spearman). There was no relationship between functional EPCs and circulating levels of TNF-α.

TABLE 7. HORMONAL AND INFLAMMATORY PROFILE OF HEALTHY PREGNANT AND PRE-ECLAMPTIC WOMEN

	Healthy pregnant women (n=19)					
	16 weeks	**24 weeks**	**32 weeks**	**37 weeks**	**Post-partum visit**	**Significance (one-way ANOVA) pregnancy 4 groups)**
Oestradiol (pmol/L)	14969±1120	33360±2268	50297±2683	62932±4503	217±67.8	p<0.0001
Progesterone (nmol/L)	178±11.2	287±15.2	671± 44	832±47.7	4±0.5	p<0.0001
sICAM-1 (ng/mL)	222±9.6	237±14.2	223±10.9	227±11.8	273±11.8	NS
TNF-α (pg/mL)*	0.12 (0-0.85)	0 (0-0.16)	0.12 (0-0.96)	0.47 (0-1.4)	0.31 (0-1.1)	NS
IL-6 (pg/mL)*	0.88 (0.23-1.8)	1.02 (0.29-1.9)	0.95 (0.44-1.3)	1.1 (0.65-1.7)	1.1 (0.6-6)	NS

	Pre-eclamptic women (n=16)			Significance		
	Pre-term (30 weeks) (n=8)	**Term (38 weeks) (n=8)**	**Post-partum visit (combined)**	**Pre-term *vs* 32 weeks**	**Term *vs* 37 weeks**	**Between post-partum groups**
Oestradiol (pmol/L)	29950±9876	70110±10715	167±36.2	p=0.009	NS	NS
Progesterone (nmol/L)	653±130.9	949±97	5±1	NS	NS	NS
SICAM-1(ng/mL)	188±14.9	203±22.9	253±24.9	NS	NS	NS
TNF-α (pg/mL)*	0 (0-0.56)	0.105 (0-0.56)	0 (0-0.35)	NS	NS	NS
IL-6 (pg/mL)*	0.92 (0.2-6)	1.055 (0.8-1.4)	0.67 (0.4-1.3)	NS	NS	NS

Data expressed as mean±SEM or *median with interquartile range. Circulating oestradiol and progesterone increase with gestation.
Plasma TNF-α, IL-6 and sICAM-1 concentrations did not vary significantly within pregnancy.
ANOVA - analysis of variance; TNF-α - tumour necrosis factor- α; IL - interleukin; sICAM-1 - soluble intercellular adhesion molecule-1; SEM - standard error of the mean; NS - not significant.

Plasma IL-6

Concentrations of plasma IL-6 were raised in pregnancy compared to healthy non-pregnant women (pooled data across the cycle, as no cyclical variation) and this persisted post-partum (pooled non-pregnant *versus* 16, 24, 32, 37 weeks and 7 weeks post-partum, $p<0.003$ for all, Mann-Whitney tests). However, concentrations of plasma, IL-6 did not vary significantly within pregnancy, nor were they different in the women with pre-eclampsia compared to the healthy pregnant women ($p>0.2$ for all, Freidman and Mann-Whitney tests; Table 7).

There was no relationship between phenotypic or functional EPCs and circulating levels of IL-6.

Plasma soluble ICAM-1

Levels of soluble ICAM-1 were greater in the healthy pregnant group, than in the healthy non-pregnant women (pooled data across the cycle, as no cyclical variation) at 24 and 37 weeks gestation (237±14.2 and 227±11.8 *versus* 202±5.9 $ngmL^{-1}$, $p<0.04$, two-tailed unpaired Student's *t*-tests). Post-partum levels of soluble ICAM-1 remained higher than in healthy pregnancy (post-partum versus 37 weeks, 273±11.8 *versus* 227±11.8 $ngmL^{-1}$, $p=0.0004$, two-tailed, paired Student's *t*-test) and higher than in the healthy non-pregnant women (post-partum *versus* pooled non-pregnant, 273±11.8 *versus* 202±5.9 $ngmL^{-1}$, $p<0.0001$, two tailed, unpaired Student's *t*-test). However, like TNF-α and IL-6, concentrations of soluble ICAM-1 did not vary significantly within pregnancy, nor were they different in the women with pre-

eclampsia compared to the healthy pregnant women ($p>0.08$ for all, one-way ANOVA with repeated measures and two-tailed unpaired Student's *t*-tests; Table 7).

Soluble ICAM-1 levels were correlated with phenotypic EPCs in samples from the 32-week visit only ($r=0.46$, $p=0.04$, $r2=0.21$, Pearson's test). There was no relationship between functional EPCs and circulating levels of soluble ICAM-1.

5.4 DISCUSSION

This study demonstrates that numbers of circulating EPCs quantified by phenotype ($CD34^+CD133^+KDR^+$ cells) vary during the menstrual cycle, peaking in the mid-follicular phase. In contrast, EPCs quantified by the functional CFU-EPC assay did not vary during the menstrual cycle, though numbers formed from samples taken in the early-follicular phase were higher than samples taken in the second and third trimesters of healthy pregnancy. During the normal menstrual cycle, mobilised $CD34^+CD133^+KDR^+$ cells may have the potential to contribute to endometrial angiogenesis during the mid-follicular phase of the cycle [Girling and Rogers, 2005]. Given the lack of correlation between EPCs quantified by phenotype or function and the levels of circulating sex hormones or inflammatory mediators measured during the menstrual cycle, this raises the possibility of an endometrial specific factor being present in mid-follicular phase endometrium, which has the potential to mobilise EPCs and/or remove them from the peripheral circulation into endometrium. There

was no difference in circulating EPCs, quantified either by phenotype or the functional CFU-EPC assay, during healthy or pre-eclamptic pregnancies.

5.4.1 EPCs AND THE MENSTRUAL CYCLE

During the normal menstrual cycle, the uterine endometrium undergoes a unique cycle of physiological angiogenesis and endothelial repair. These processes begin in the proliferative and continue into the secretory phase of the cycle and are under tight hormonal and inflammatory control [Jabbour *et al*, 2006]. Previously, endometrial angiogenesis was thought to occur primarily from new vessels sprouting via recruitment of local endothelial cells from neighbouring blood vessels [Risau and Flamme, 1995; Gambino *et al*, 2002]. However recently, an alternative or complementary mechanism involving circulating EPCs has been proposed in the control and regulation of endometrial angiogenesis [Mints *et al*, 2008]. In support, in mice, an average 6% of endometrial endothelial cells were found to be donor derived after a haematological stem cell transplant [Bratincsak *et al*, 2007].

The present study is the first to investigate the influence of the menstrual cycle on both phenotypic and functional EPCs. The finding that $CD34^+CD133^+KDR^+$ EPCs peak during the mid-follicular phase differs from two recent studies of EPCs during the menstrual cycle [Matsubara *et al*, 2006; Fadini *et al*, 2008]. A variety of factors may account for these seemingly discrepant findings, but in particular the differing methodologies employed and timing of sampling Fadini *et al* [2008] demonstrated a similar 2-fold increase in $CD34^+KDR^+$ numbers during the menstrual cycle, but the peak seemed to occur in the peri-ovulatory phase rather than earlier during the

follicular phase as in our study. However, Fadini *et al* [2008] did not specify the day of sampling so it is possible that this peak occurred between days 6 to 12 of the menstrual cycle, which would therefore be entirely consistent with our findings. In the cross-sectional study by Matsubara *et al* [2006], EPCs were increased in the luteal phase compared to the follicular phase of the cycle in women. The discrepancy in findings is most likely due to the different methodology and study design employed. They quantified the proliferative capacity of EPCs using adherent peripheral blood mononuclear cells that tested positive for AcLDL and lectin after 7 days in culture, and did not use the EPC-CFU assay described here. Moreover, although Matsubara *et al* [2006] compared EPCs during the follicular and luteal phase of the cycle, it is again unclear on which day the samples were taken in their cross-sectional study. From this longitudinal study, in which samples were taken on four consecutive time-points within a single cycle, there are clear differences in concentrations of $CD34^{+}CD133^{+}KDR^{+}$ EPCs depending on the day of sampling. This variation will be magnified if a broader time window of sampling was used, and by the use of multiple subjects because of the potential for inter-subject variability.

5.4.2 EPCs AND HEALTHY PREGNANCY

The results in this chapter demonstrate that neither $CD34^{+}CD133^{+}KDR^{+}$ EPCs nor CFU-EPCs vary throughout healthy pregnancy. In healthy pregnant women, the formation of CFU-EPCs, was lower in samples from women in the early-follicular phase of the menstrual cycle. This finding is consistent with the inflammatory state of pregnancy. Although the exact phenotype of EPCs is still under debate (discussed below) and varies widely in the literature, recent reports demonstrate that induced

human endotoxaemia decreases EPCs, measured within the EPC-CFU assay [Spiel *et al*, 2008].

However, our findings contrast to nearly all the other studies performed in pregnancy. The first published studies employed a cross-sectional design [Gussin *et al*, 2002; Sugawara *et al*, 2005a; Matsubara *et al,* 2006] and demonstrated both increases [Sugawara *et al*, 2005a] and decreases [Matsubara *et al*, 2006] of EPCs with gestation. The only other longitudinal study, by Buemi *et al* [2007], of seven women, reported an increase in double positive ($CD133^+/KDR^+$) cells. Our findings are supportive of the most recent study by Savvidou *et al* [2008] who also found no change in numbers of EPCs with gestation, and lower levels in healthy pregnant women compared to non-pregnant women, though they used different methods, performing flow cytometry on an adherent population of mononuclear cells after 7 days in culture.

It is difficult to make straightforward comparisons between all these studies, as different methods have been used to quantify putative EPCs. In particular, discordance between flow cytometry and cell culture will be discussed below. However, a limitation of all of these studies, except Buemi *et al* [Buemi *et al* 2007], is also the cross-sectional study design. Many factors affecting circulating EPC numbers were not described for the study subjects. A strength of this current study therefore is its prospective design following women through gestation and post-partum. This has demonstrated that in pregnancy, CFU-EPCs are lower than in the follicular phase of the menstrual cycle. Due to the difficulties in recruiting women

for a longitudinal study before they have had a booking ultrasound scan, we were unable to obtain data on circulating EPCs in the first trimester. If EPCs play an active role within the uterus at the time of placentation, our study would have missed this sampling opportunity.

5.4.3 EPCs AND PRE-ECLAMPSIA

The finding that $CD34^+CD133^+KDR^+$ EPCs and CFU-EPC numbers are no different in women with pre-eclampsia compared to healthy pregnant women at similar gestations partly supports and differs from the only other studies of EPCs in pre-eclamptic women [Sugawara *et al*, 2005b; Matsubara *et al*, 2006]. As with the studies in normal pregnancy, the published data are conflicting. Sugawara *et al* demonstrated decreased numbers of CFU-EPCs and increased senescence of EPCs in patients with pre-eclampsia compared to gestationally matched controls [Sugawara *et al* 2005b]. In contrast, Matsubara *et al* [2006] reported no difference in the number of circulating $CD34^+CD133^+KDR^+$ EPCs measured by flow cytometry in pre-eclamptic women, supporting this similar finding. However, they also performed culture of fibronectin-adherent mononuclear cells, not the non-adherent fraction as in the CFU-EPC assay. In their method, the samples from women with pre-eclampsia resulted in more endothelial-like cells, which had increased proliferative response following stimulation with TNF-α and angiotensin II compared with cells from women without pre-eclampsia [Matsubara *et al*, 2006]. This increase may be a physiological response to ischaemia in the placenta and other organs, similar to that seen in myocardial infarction [Leone *et al*, 2005; Massa *et al*, 2005]. These studies

are not easily comparable because of the different methods used, and it is difficult to draw a conclusion from the discordant observations.

Although the CFU-EPC assay quantifies colony forming units, different assays are emerging, which further assess cell function, proliferative ability and endothelial tube formation. It is likely that EPC function is more important than quantity, and ideally a subsequent study of EPCs in pre-eclampsia would assess number and function prospectively prior to the onset of disease.

5.4.4 Quantification of EPCs

The exact phenotype of EPCs has been debated since the original publication by Asahara *et al* [1999] in which it was demonstrated that $CD34^+$ cells extracted from circulating peripheral blood formed new blood vessels in culture and contributed to angiogenesis in a mouse model of ischaemia. The purity of this cell population was only 15% raising the possibility that a variety of other circulating cell types may also have angiogenic potential. The current consensus is that mononuclear cells co-expressing CD34, CD133, and KDR (so-called 'triple positive' cells) constitute the most likely phenotype of circulating endothelial progenitors. However, these cells are rare in normal peripheral blood, constituting between 0.01% and 0.0001% of the circulating leukocyte population, making their accurate quantification by flow cytometry difficult. As a result, assays based on the quantification of functional populations of EPCs with colony forming activity in cell culture (CFU-EPCs) have emerged as alternatives [Hill *et al*, 2003].

However, the validity of the CFU-EPC assay in quantifying EPCs has recently been challenged by studies showing that the CFU-EPC cells are derived from monocyte-like cells and not the circulating $CD34^+$ haemangioblast [Prater *et al*, 2007; Rohde *et al*, 2007; Yoder *et al*, 2007]. Recent work, performed by other members of our group, is consistent with that of Rhode *et al* [2007] where it has been demonstrated that there are substantial numbers of CD14 expressing cells in the non-adherent population replated at 48 hours in the CFU-EPC assay. This expression of CD14 is gradually reduced during culture in endothelial medium. Using flow cytometric sorting, CFU-EPCs only develop in the presence of CD14 cells. Isolated $CD34^+$ or $CD133^+$ cells are not able to form CFU-EPC in this assay (personal communication, Dr Olga Tura, The Univeristy of Edinburgh).

Therefore, a strength of this study design was the simultaneous measurement of $CD34^+CD133^+KDR^+$ cells using flow cytometry and quantification of colony forming units in the CFU-EPC assay. Both remain the most established and widely used techniques in the field, though our group [Tura *et al*, 2007] and others have now confirmed that these techniques are measuring quite different cell populations.

The finding of a lack of correlation between concentrations of $CD34^+CD133^+KDR^+$ EPCs and EPCs quantified by the CFU-EPC assay is therefore important. Currently, these two methods are used interchangeably in clinical studies and are in general thought to be measuring the same endothelial progenitor derived from $CD34^+$ pluripotent cells using different techniques. This is clearly not the case as our study demonstrates. Thus, cells quantified by flow cytometry (phenotype) and cell culture

(CFU-EPCs) are likely to have different biological functions and progenitor potential [Tura *et al*, 2007]. Ingram *et al* [2004] described a new EPC population in human umbilical cord blood called the 'late outgrowth endothelial progenitor cell' with a high proliferative capacity and the ability to express endothelial markers and form tubules *in vitro* [Ingram *et al*, 2004]. This population arises from $CD34^+$ cells. Thus, the $CD34^+CD133^+KDR^+$ EPCs may correlate with this late-outgrowth assay.

5.4.5 HORMONAL REGULATION OF EPCS

This research has demonstrated that $CD34^+CD133^+KDR^+$ EPCs peak during the mid-follicular phase of the menstrual cycle. Neither healthy pregnancy, nor pre-eclampsia appeared to affect the absolute concentrations of EPCs. Colony forming unit-endothelial progenitor cell formation was not different across the menstrual cycle, but was greater in the early follicular phase than in healthy pregnancy. Again, neither gestation, nor pre-eclampsia appeared to further affect CFU-EPC formation. In this study, these effects do not appear to be driven by the circulating oestradiol or progesterone. The lack of correlation of EPCs with serum oestradiol concentrations differs from the study by Sugawara *et al* [2005a] in which they found that during pregnancy, there was a clear correlation between oestradiol and EPCs quantified by a modified CFU-EPC assay [Sugawara *et al.* 2005a]. However, unlike their study the results presented here do not demonstrate any change within pregnancy in CFU-EPCs or $CD34^+CD133^+KDR^+$ EPCs. There was a positive correlation between number of CFU-EPCs and circulating oestradiol and progesterone concentrations in samples from the 24-week visit only. This correlation did not exist for $CD34^+CD133^+KDR^+$ EPCs and it is difficult to understand the significance of this

relationship at a single time point in pregnancy. Differences in methodology are most likely to account for the differences between this study and that of Sugawara *et al* [2005a].

It was surprising not to find a consistent relationship between EPC concentrations and oestradiol, as oestrogens are known to have vasculoprotective effects, in part due to increasing production of nitric oxide and by decreasing reactive oxygen species [Mendelsohn and Karas, 1999]. Outwith pregnancy, oestrogens mobilise EPCs from the bone marrow *in vivo* and inhibit the senescence of EPCs and stimulate VEGF production *in vitro* [Strehlow *et al*, 2003; Imanishi *et al*, 2005b]. These findings suggest that factors other than oestrogens may be involved in EPC mobilisation in non-pregnant and pregnant women.

5.4.6 EPCs AND INFLAMMATORY MEDIATORS

In this study, there was no consistent correlation between concentrations of the circulating inflammatory mediators studied and EPCs. A number of factors may explain this. First, unlike $CD34^+CD133^+KDR^+$ EPCs in the menstrual cycle, there was no variation in $CD34^+CD133^+KDR^+$ EPCs in healthy pregnancy or pre-eclampsia. In addition, CFU-EPC formation did not vary across the cycle, within healthy pregnancy or in pre-eclampsia. Secondly, there was no cyclical variation in concentration of the inflammatory mediators IL-6, soluble ICAM-1 and VEGF over the menstrual cycle. Although, there was a decrease in soluble TNF-α concentration over the cycle and a weak correlation with numbers of $CD34^+CD133^+KDR^+$ EPCs, the absolute levels of TNF-α were almost ten times lower than in conditions such as

rheumatoid arthritis and cardiac failure, where strong correlations with EPCs have been demonstrated [Valgimigli *et al*, 2004; Grisar *et al*, 2007]. Moreover, the small numbers in our menstrual cycle study may also be a contributing factor.

Circulating levels of IL-6 and soluble ICAM-1 were higher in pregnancy than in healthy non-pregnant women, reflecting the systemic inflammatory state, and this persisted post-partum. TNF-α though was not significantly different in pregnancy compared to the non-pregnant state. None of the inflammatory markers measured increased with gestation, and perhaps most surprisingly, none were affected by pre-eclampsia. Our women were well matched gestationally and fulfilled ISSHP criteria for the diagnosis of pre-eclampsia. A recent study that reported no difference in the circulating concentrations of seventeen cytokines between pre-eclamptic and gestationally matched women [Brewster *et al*, 2008].

There were some isolated correlations in healthy pregnancy, negative between plasma TNF-α and $CD34^+CD133^+KDR^+$ EPCs at 16 and 24 weeks gestation and a positive correlation between soluble ICAM-1 and $CD34^+CD133^+KDR^+$ EPCs at 32 weeks gestation, the significance of these is uncertain. Therefore, local rather than systemic levels of inflammatory mediators may control mobilisation of EPCs. Mobilisation of EPCs in response to increased local but not systemic concentrations of VEGF and SDF-1 has recently been demonstrated in animal and human limb ischaemia models [Hur *et al*, 2007; Oh *et al*, 2007]. Within the menstrual cycle, this study measured systemic and not local levels of VEGF, which may account for the lack of correlation between VEGF and EPCs. Finally, other factors thought to be

involved in regulation of endometrial angiogenesis including the angiopoietins [Girling and Rogers, 2005; Saito *et al*, 2007], may play a role in EPC mobilisation and recruitment [Mints *et al*, 2007] during the mid-follicular phase of the menstrual cycle and possibly pregnancy.

5.5 SUMMARY

In summary, during the normal menstrual cycle there is cyclical variation of phenotypic triple positive EPCs. Their peak in the mid-follicular phase suggests a potential role for circulating EPCs in the normal cycle of physiological angiogenesis and repair of the uterine endometrium. It would be of interest to determine whether pathologies such as heavy menstrual bleeding and endometriosis [Mints *et al*, 2007] are associated with abnormalities of EPC number or function. Colony forming unit-endothelial progenitor cells but not 'triple positive' EPCs were higher in the follicular phase of the menstrual cycle than in pregnancy, though there was no variation in EPCs measured by either assay with pre-eclampsia; suggesting that systemic involvement of these cells is affected by pregnancy, but not altered in pre-eclampsia. This work has mainly focused on quantification of these cells. Areas of interest for future studies would examine their role in the first trimester of pregnancy, in women prior to the development of pre-eclampsia and further evaluation of their functional ability.

CHAPTER 6

THE INFLUENCE OF THE MENSTRUAL CYCLE, HEALTHY PREGNANCY AND PRE-ECLAMPSIA ON ARTERIAL STIFFNESS

Robb AO, Mills NL, Din JD, Smith IBJ, Paterson F, Newby DE, Denison FC. Influence of the menstrual cycle, pregnancy and preeclampsia on arterial stiffness. *Hypertension* 2009;**53**:952-958.

6.1 INTRODUCTION

Arterial stiffness is a key determinant of central aortic pressure and is an independent predictor of adverse cardiovascular outcomes and organ damage [Laurent *et al*, 2001; Wilkinson *et al*, 2001a]. Female gender affects arterial stiffness, mediated in part via the influence of both oestrogen and progesterone on arterial structure and function [Natoli *et al*, 2005]. In the pre-pubertal and post-menopausal years when female sex steroids are low, women have stiffer arteries than age-matched men [Waddell *et al*, 2001; Ahimastos *et al*, 2003]. During the reproductive years, female sex steroids fluctuate cyclically during the menstrual cycle and increase dramatically in pregnancy. The initial effect of pregnancy increasing arterial compliance is well documented in both animal and human studies [Hart *et al*, 1986; Poppas *et al*, 1997; Slangen *et al*, 1997; Edouard *et al*, 1998; Smith *et al*, 2004; Mersich *et al*, 2005]. However data are conflicting, concerning the effect of the menstrual cycle, (where circulating concentrations of sex steroids are lower), and the effect of later gestation on arterial compliance.

Augmentation index and PWV are the principal measures of central arterial pressure and stiffness that can be determined noninvasively using applanation tonometry. Analysis of peripheral pressure waveforms allows the derivation of central arterial pressure and its augmentation by pulse wave reflection; often expressed as the AI and provides a measure of systemic arterial stiffness. Pulse wave velocity is the 'gold standard' technique for assessing large vessel stiffness and is usually performed at

the carotid and femoral or carotid and radial arteries to measure aortic and brachial stiffness respectively.

Within normotensive pregnancy, PWV is more closely associated with low birth-weight than mean arterial pressure, suggesting that arterial stiffness may represent maternal adaptation to pregnancy better than blood pressure [Elvan-Taspinar *et al*, 2005]. Pre-eclampsia is a common hypertensive complication of pregnancy, which causes significant maternal and foetal morbidity. Inadequate cardiovascular adaptation in early pregnancy may predate its clinical presentation [De Paco *et al*, 2008], and it is associated with an increased long-term risk of maternal cardiovascular disease. Understanding the relationship between pre-eclampsia and arterial stiffness may therefore not only inform understanding of its pathogenesis, but may also increase our understanding of the association between pre-eclampsia and later cardiovascular disease.

The aim of the research presented in this chapter was to evaluate the effect of the menstrual cycle, normal pregnancy and pre-eclampsia on central and systemic arterial stiffness.

6.2 METHODS

All equipment used is detailed in Appendix 1.

6.2.1 SUBJECT RECRUITMENT

Women were identified, recruited and consented to this study (study 2, Reference number 05/S1104/48), as described in section 2.1.

Non-pregnant women

Healthy pre-menopausal non-smoking nulliparous women (n=16) with at least a 2-month history of normal regular menstrual cycles were recruited to the study. Exclusion criteria are listed in section 2.1.2.

Pregnant women

Healthy, non-smoking primigravida women with an uncomplicated pregnancy (n=30) or with pre-eclampsia (n=16) were recruited in the first trimester of pregnancy or at diagnosis respectively. Women were given a minimum of 24 hours to consider participating in the study. Exclusion criteria are listed in section 2.1.3.

6.2.2 VISIT SCHEDULE

Non-pregnant women

Women attended for four visits during a single menstrual cycle (D 1-3 (early-follicular), D 6-8 (mid-follicular), D 13-15 (peri-ovulatory) and D 20-22 (mid-luteal)).

Pregnant women

Women with uncomplicated pregnancies attended for four visits during pregnancy at 16, 24, 32 and 37 weeks and at 7 weeks post-partum. Women with pre-eclampsia attended following diagnosis and at 7 weeks post-partum.

6.2.3 STUDY PROTOCOL

At each visit, all women abstained from alcohol and caffeine for 12 hours and fasted for 4 hours prior to attendance. Height and weight were measured to allow calculation of BMI. Women with pre-eclampsia continued to take their antihypertensive medication as prescribed. This is detailed in section 6.3.2. All studies were performed in a quiet, temperature controlled room with women resting in the supine position or 30° left lateral position for pregnant women (to avoid inferior vena cava compression by the uterus). All subsequent measurements were done in the supine position. Following a 30-minute rest period (during which venepuncture was performed as detailed in chapters 4 and 5), blood pressure and heart rate were recorded in duplicate using an automated sphygmomanometer validated for use in pregnancy and pre-eclampsia [Reinders *et al*, 2005].

Pulse wave analysis

This was performed on all women at every visit as detailed in section 2.2.1.

Pulse wave velocity

Pulse wave velocity was performed in the early-follicular visit for the non-pregnant women and at every visit (pregnant and post-partum) for the healthy pregnant and pre-eclamptic women as detailed in section 2.2.2.

6.2.4 Measurement of circulating hormones

Serum concentrations of oestradiol and progesterone were measured in all women. Lutenizing hormone, and FSH were also measured in the healthy non-pregnant women. All hormones were measured by automated chemiluminescent microparticle assays as described in section 2.9.1-4.

6.2.5 Statistical analysis

Continuous variables are reported as mean±SEM for parametric data. Statistical analyses were performed using paired and unpaired two-tailed Student's *t*-tests. For multiple comparisons, one-way ANOVA with repeated measures was used. All calculations were performed using GraphPad Prism, (GraphPad Software, USA). Statistical significancc was taken at 5%.

6.3 RESULTS

6.3.1 Subject characteristics

Non-pregnant women

In six of sixteen non-pregnant women, ovulation could not be confirmed leaving a final study group of ten women with a confirmed regular ovulatory cycle (cycle length 28±0.4 days; luteal phase serum progesterone >30 nmol/L).

Pregnant women

In the healthy pregnant group, four of thirty women developed complications in their pregnancy, for example intra-uterine growth restriction, pre-term labour or pre-eclampsia and a further four were unable to complete the full schedule of visits, leaving a final study group of twenty-two women for analysis. In the group of pre-eclamptic women, one woman could not complete the study, leaving a final study group of fifteen women. Women were categorised according to gestation at presentation, into pre-term (n=7; mean 30 (range 24-34 weeks)) and term (n=8; mean 38 (range 36-40 weeks)). As anticipated, women with pre-eclampsia were delivered earlier than women with uncomplicated pregnancies and had offspring of lower birth weight The mean gestation at delivery for women in the pre-term and term group was 30.3 weeks (range 24-35.8) and 38.6 weeks (range 36.4-40.4), respectively. Non-pregnant and pregnant groups were well matched for maternal age and BMI. However, pregnant women were shorter in height than the non-pregnant group (Table 8).

TABLE 8. BASELINE CHARACTERISTICS OF STUDY PARTICIPANTS

	Healthy non-pregnant group	Healthy pregnant group	Pre-eclamptic group (n=15)	
	(n=10)	(n=22)	Pre-term (n=7)	Term (n=8)
Age (years)	31±2	30±1	30±4	30±2
Height (m)	1.70±0.02	1.65±0.02*	1.63±0.02*	1.62±0.02*
Weight (Kg)	68±3	73±3	66±4	75±4
Body Mass Index (kg m^{-2})	23±1	27±1	25±2	29±1†
Gestation at delivery (weeks)	N/A	41±0.3	30±1.4 ‡	39±0.4 ‡
Birth weight (g)	N/A	3497±112	1371±222§	3231±252

Data expressed as mean±SEM. Body mass index was recorded during first trimester for pregnant women.
* $p<0.05$ and † $p\leq0.0009$ *versus* non-pregnant group, ‡ $p\leq0.0003$ *versus* healthy pregnant group and § $p<0.0001$ *versus* healthy pregnant and term pre-eclamptic groups, (two-tailed unpaired Student's *t*-tests).
SEM – standard error of the mean.

There were no differences in systolic and diastolic blood pressure at booking between women with healthy and pre-eclamptic pregnancies (pre-term and term *versus* healthy pregnant, both $p>0.1$). However, as expected, women with pre-eclampsia had higher systolic and diastolic blood pressures. At the post-partum visit, although blood pressure had returned to within the normal range in women who had pre-eclampsia, it was still higher than in those women who had had an uncomplicated pregnancy ($p\leq0.004$, two-tailed unpaired Student's *t*-test; Table 9).

6.3.2 Medication use

In women with pre-term pre-eclampsia, five were taking regular labetalol and nifedipine, one was taking regular methyldopa and nifedipine, and one was receiving no anti-hypertensive therapy. Six of these women received antenatal betamethasone. In women with term pre-eclampsia, one was taking regular labetalol with the remaining seven women not receiving anti-hypertensive therapy. None received antenatal betamethasone. Post-partum, out of the original fifteen women who had developed pre-eclampsia, only three women were taking labetalol, and one, methyldopa.

6.3.3 Circulating hormones

Within the menstrual cycle, there was normal cyclical variation in circulating pituitary and ovarian hormones with peri-ovulatory peaks in serum LH and FSH concentrations, and peri-ovulatory and mid-luteal peaks in serum oestradiol and progesterone concentrations respectively (Table 10).

TABLE 9. HAEMODYNAMIC VARIABLES OF BOTH PREGNANT GROUPS

Haemodynamic Variables	Healthy Pregnant Group (n=22) 16 weeks	24 weeks	32 weeks	37 weeks	Post-partum visit	Significance Within pregnancy (4 time points, 16 to 37 weeks)
Heart rate (bpm)	69±2	72±2	77±2	77±2	59±1	p<0.0001
Peripheral SBP, mmHg	113±2	111±1	113±1	117±1	113±1	p=0.004
Peripheral DBP, mmHg	65±1	65±1	70±1	76±1	69±1	p<0.0001
Peripheral PP, mmHg	48±2	46±2	43±1	41±1	42±2	p=0.003
Central SBP, mmHg	93±2	92±1	95±1	103±2	98±2	p<0.0001
Central DBP, mmHg	64±1	64±1	69±1	76±1	70±1	p<0.0001
Central PP, mmHg	30±1	28±1	26±1	27±1	29±2	p=0.13
Mean Arterial Pressure, mmHg	81±1	80±1	84±1	90±1	84±1	p<0.0001

Haemodynamic Variables	Pre-eclamptic Group (n=15) Pre-term (30 weeks) (n=7)	Term (38 weeks) (n=8)	Post-partum visit	Significance Pre-term *vs* 32 weeks	Term *vs* 37 weeks	Post-partum comparisons
Heart rate (bpm)	67±3	76±5	65±3	p=0.02	p=0.8	p=0.02
Peripheral SBP, mmHg	140±3	146±2	123±3	p<0.0001	p<0.0001	p=0.002
Peripheral DBP, mmHg	87±3	95±2	77±3	p<0.0001	p<0.0001	p=0.004
Peripheral PP, mmHg	52±4	52±4	45±3	p=0.004	p=0.001	p=0.4
Central SBP, mmHg	126±4	135±3	111±3	p<0.0001	p<0.0001	p=0.0006
Central DBP, mmHg	89±3	97±2	79±3	p<0.0001	p<0.0001	p=0.002
Central PP, mmHg	37±2	38±3	33±2	p<0.0001	p=0.0002	p=0.2
Mean Arterial Pressure, mmHg	105±2	113±2	92±2	p<0.0001	p<0.0001	p=0.001

Data expressed as mean±SEM.
SBP - systolic blood pressure; DBP - diastolic blood pressure; PP - pulse pressure; bpm - beats per minute; SEM - standard error of the mean.

TABLE 10. HORMONAL PROFILE OF NON-PREGNANT WOMEN

	Day of menstrual cycle				
	1-3	**6-8**	**13-15**	**20-22**	**Significance**
Day at sampling	2±0.2	7 ±0.2	14±0.3	21±0.2	
LH (UL^{-1})	5±0.4	7±0.4	32±7.2	5±0.5	$p<0.0001$
FSH (UL^{-1})	6±0.4	6±0.4	9±1.9	3±0.2	$p=0.002$
Oestradiol ($pmol\ L^{-1}$)	180±21.1	318±68.4	1342±274.4	827±93.4	$p<0.0001$
Progesterone ($nmol\ L^{-1}$)	4±0.3	3±0.2	7±2.1	47±4.5	$p<0.0001$

Data expressed as mean±SEM. Circulating LH, FSH, oestradiol and progesterone varied significantly throughout the menstrual cycle.
LH - lutenising hormone; FSH - follicle stimulating hormone; SEM - standard error of the mean.

Expected increases with gestation were observed in pregnant women in serum oestradiol and progesterone concentrations (Table 11). Pre-term pre-eclamptic women had lower serum oestradiol levels than healthy pregnant women at 32 weeks gestation (29950±9876 *versus* 49222±2444 pmol L^{-1}, p=0.009, two-tailed unpaired Student's *t*-test).

TABLE 11. HORMONAL PROFILE OF THE HEALTHY PREGNANT AND PRE-ECLAMPTIC WOMEN

	Healthy Pregnant Group (n=22)					
	16 weeks	**24 weeks**	**32 weeks**	**37 weeks**	**Post-partum visit**	**Significance over pregnancy (4 groups)**
Oestradiol (pmol L^{-1})	14995±1057	32350±2108	49222±2444	61385±4306	210±47.1	p<0.0001
Progesterone (nmol L^{-1})	178±9.9	278±14	670±38.6	813±44.5	4±0.5	p<0.0001

	Pre-eclamptic Group (n=15)					
	Pre-term (30 weeks) (n=7)	**Term (38 weeks) (n=8)**	**Post-partum visit (combined)**	**Significance pre-term *vs* 32 weeks**	**Significance term *vs* 37 weeks**	**Significance between post-partum groups**
Oestradiol (pmol L^{-1})	29950±9876	70110±10715	167±36.2	p=0.009	NS	NS
Progesterone (nmol L^{-1})	653±130.9	949±97	5±1	NS	NS	NS

Data expressed as mean±SEM. Circulating oestradiol and progesterone increase with gestation.
SEM - standard error of the mean; NS - not significant.

6.3.4 EFFECT OF THE MENSTRUAL CYCLE ON SYSTEMIC ARTERIAL STIFFNESS

In women with confirmed ovulatory cycles, systemic arterial stiffness, as measured by AI, varied over the menstrual cycle ($p=0.03$, one-way ANOVA with repeated measures) with a fall in the luteal phase compared to the peri-ovulatory phase (3.5±1.8% *versus* 9.9±1.8%, $p<0.05$ Bonferroni's post-test; Figure 34a). There were no changes in any other recorded haemodynamic variables throughout the menstrual cycle (Table 12). There was no correlation between AI and serum oestradiol or progesterone at any time point in the cycle.

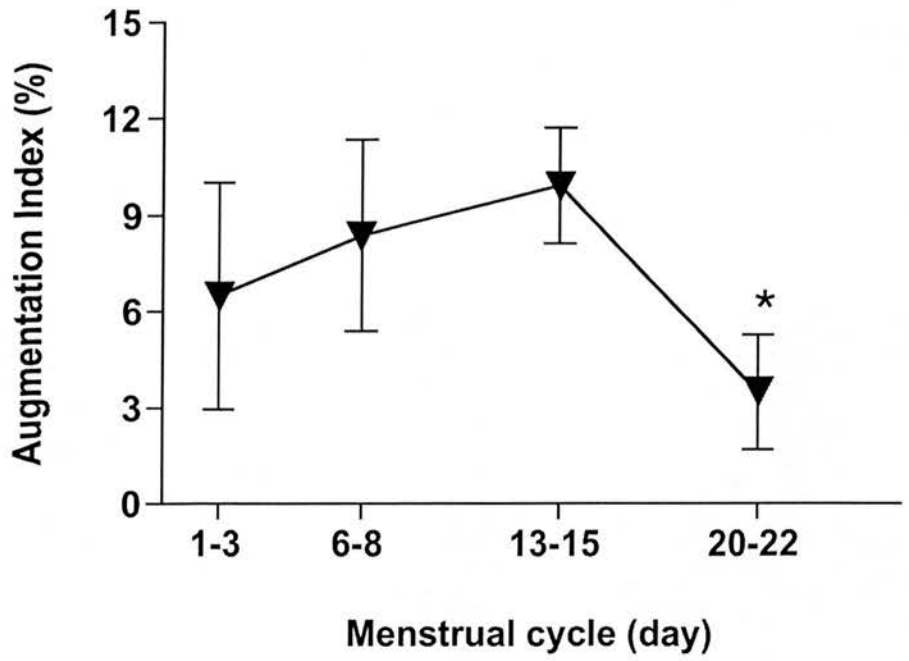

Figure 34a. Variation in augmentation index throughout the menstrual cycle. Data (n=10) are reported as mean±SEM. Augmentation index varied during the menstrual cycle ($p=0.03$, one-way ANOVA with repeated measures), with a fall in the luteal phase compared to the peri-ovulatory phase, (days 20-22 *versus* 13-15, $p<0.05$ Bonferroni's post-test.)
ANOVA - analysis of variance.

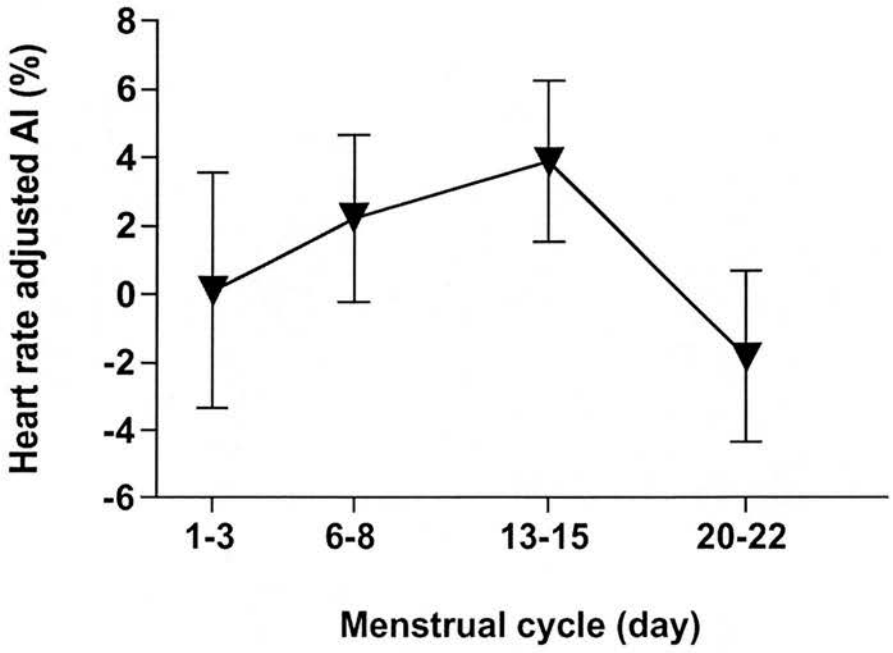

Figure 34b. Variation in heart rate adjusted augmentation index throughout the menstrual cycle. Data (n=10) are reported as mean±SEM. There was a trend towards a reduction in heart rate adjusted Augmentation index, with a fall in the luteal phase compared to the peri-ovulatory phase, (p=0.07, one-way ANOVA with repeated measures). ANOVA - analysis of variance.

TABLE 12. HAEMODYNAMICS OF THE HEALTHY NON-PREGNANT WOMEN

	Day of menstrual cycle				
	1-3	**6-8**	**13-15**	**20-22**	**Significance (one-way ANOVA)**
Heart rate (bpm)	60±2	61±3	61±3	63±3	p=0.5
Peripheral SBP, mmHg	110±2	108±1	108±3	110±2	p=0.7
Peripheral DBP, mmHg	67±2	68±1	68±2	67±2	p=0.9
Peripheral PP, mmHg	42±2	39±2	41±1	41±2	p=0.7
Central SBP, mmHg	96±2	93±1	94±2	93±2	p=0.3
Central DBP, mmHg	68±1	67±2	67±2	68±2	p=1
Central PP, mmHg	28±1	26±1	27±1	25±1	p=0.5
Mean Arterial Pressure, MmHg	81±2	81±1	81±2	81±2	p=1

Data expressed as mean±SEM. SBP - Systolic blood pressure; DBP - Diastolic blood pressure; PP - Pulse pressure; bpm - beats per minute; SEM - standard error of the mean.

6.3.5 EFFECT OF NORMAL PREGNANCY ON SYSTEMIC ARTERIAL STIFFNESS

Augmentation index, was adjusted for heart rate (calculated at a heart rate of 75 bpm) due to variation in heart rate during pregnancy and post-partum ($p<0.0001$ for all, unpaired Student's *t*-test). Heart rate adjusted AI varied with gestation in normal pregnancy ($p<0.0001$, one-way ANOVA with repeated measures; Figure 35) rising towards term (16 weeks *versus* 37 weeks, 24 weeks *versus* 37 weeks and 32 weeks *versus* 37 weeks, all $p<0.01$, Bonferroni's post-tests). Moreover, heart rate-adjusted AI was persistently elevated at 7 weeks post-partum compared to 16 weeks gestation (8.7±1.9% *versus* -3.0±2.5%, $p=0.0002$, two-tailed paired Student's *t*-test). There was no correlation between AI and serum oestradiol or progesterone at any time point in pregnancy.

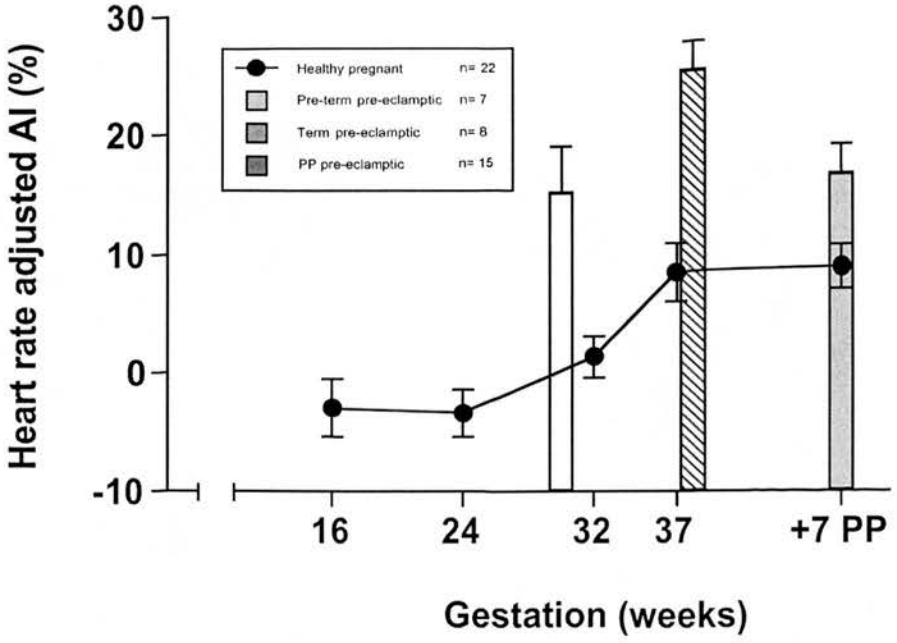

Figure 35. The effect of pregnancy, gestation and pre-eclampsia on augmentation index (adjusted for heart rate). Data reported as mean±SEM. Heart rate adjusted augmentation index varied with gestation in normal pregnancy ($p<0.0001$) rising towards term ($p<0.01$) and was elevated at 7 weeks post-partum compared to 16 weeks gestation ($p=0.0002$). Compared to gestation matched controls, augmentation index was raised in both pre-eclamptic groups (pre-term and term, both $p\leq0.001$) and remained elevated post-partum ($p=0.02$). There was no difference in augmentation index, between the two pre-eclamptic groups ($p=0.05$).
AI - augmentation index; PP - post-partum; SEM - standard error of the mean.

6.3.6 Effect of normal pregnancy on central arterial stiffness

Both carotid-femoral and carotid-radial PWV varied with gestation in normal pregnancy (both $p=0.01$, one-way ANOVA with repeated measures; Figures 36 and 37, respectively). Carotid-femoral PWV increased from 24 weeks to 7 weeks post-partum (5.0±0.2 m/s *versus* 5.5±0.2 m/s, $p=0.0008$, two-tailed paired Student's *t*-test). Carotid-radial PWV rose from 16 and 24 weeks to term (16 weeks *versus* 37 weeks, and 24 weeks *versus* 37 weeks, both 6.4±0.2 m/s *versus* 7.0±0.2 m/s, $p<0.05$ Bonferroni's post-tests), and values at 7 weeks post-partum were not

different to those at term (post-partum *versus* 37 weeks, 6.6±0.2 m/s *versus* 7.0±0.2m/s, p=0.07, two-tailed paired Student's *t*-tests). There was no correlation between carotid-femoral or carotid-radial PWV and serum oestradiol or progesterone concentrations at any time point in pregnancy.

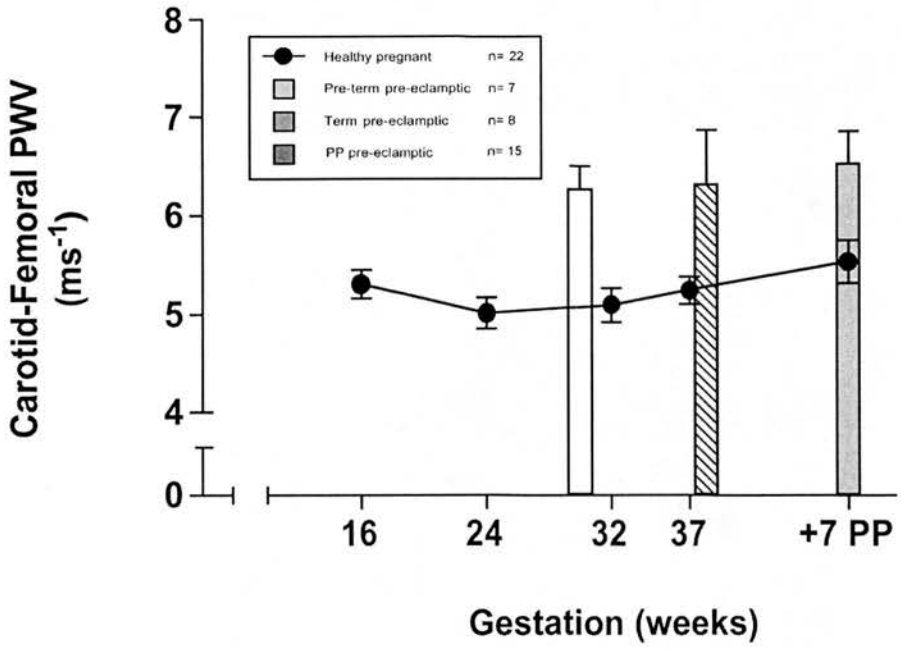

Figure 36. The effect of pregnancy, gestation and pre-eclampsia on carotid-femoral PWV. Data reported as mean±SEM. Carotid-femoral PWV varied with gestation in normal pregnancy (p=0.01). Compared to gestation matched controls, carotid-femoral PWV was raised in both pre-eclamptic groups (pre-term and term, both p≤0.01) and remained elevated post-partum (p=0.01). There was no difference in carotid-femoral PWV between the two pre-eclamptic groups.
PP - post-partum; PWV - pulse wave velocity; SEM - standard error of the mean.

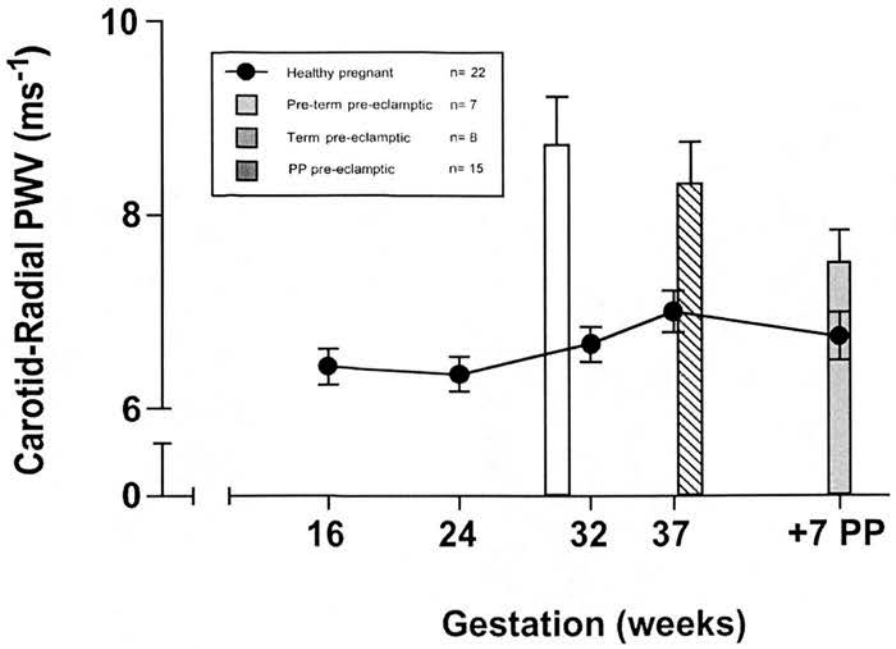

Figure 37. The effect of pregnancy, gestation and pre-eclampsia on carotid-radial PWV. Data reported as mean±SEM. Carotid-radial PWV varied with gestation during normal pregnancy ($p=0.01$). Compared to gestation matched controls, carotid-radial PWV was raised in both pre-eclamptic groups (pre-term and term, both $p \leq 0.006$). There was no difference in carotid-radial PWV, between the two pre-eclamptic groups in pregnancy.
PP - post-partum; PWV - pulse wave velocity; SEM - standard error of the mean.

6.3.7 Effect of pre-eclampsia on systemic and central arterial stiffness

All haemodynamic variables differed between the women with pre-eclampsia and the healthy pregnant women at similar gestations, apart from heart rate in women with term pre-eclampsia (Table 9).

Augmentation index, carotid-femoral and carotid-radial PWV were raised in women with both pre-term and term pre-eclampsia compared to gestationally matched women with uncomplicated pregnancies (AI pre-term 15.1±3.9% *versus* 1.3±1.7% and term 25.1±2.5% *versus* 8.4±2.4%, $p \leq 0.001$ for both, two-tailed unpaired Student's *t*-tests (Figure 35); carotid-femoral PWV pre-term 6.3±0.2 m/s *versus* 5.1±0.2 m/s and term 6.3±0.6 m/s *versus* 5.2±0.1 m/s, $p \leq 0.01$ for both, two-tailed unpaired Student's *t*-tests (Figure 36) and carotid-radial PWV pre-term 8.7±0.5 m/s *versus* 6.7±0.2 m/s and term 8.3±0.4 m/s *versus* 7.0±0.2 m/s, $p \leq 0.006$ for both, two-tailed unpaired Student's *t*-tests (Figure 37)). There was no difference in AI, carotid-femoral or carotid-radial PWV between women with pre-term or term pre-eclampsia either antenatally or at the post-partum visit (all $p > 0.05$). At the post-partum visit, despite blood pressure returning to within the normal range, AI and carotid-femoral PWV remained elevated at 7 weeks compared to women with uncomplicated pregnancies (16.2±2.5% *versus* 8.7±1.9% and 6.5±0.3 m/s *versus* 5.5±0.2 m/s respectively, $p \leq 0.02$ for both, two-tailed unpaired Student's *t*-tests (Figures 35 and 36)). In contrast, there was no difference in carotid-radial PWV by 7 weeks post-partum between women with pre-eclampsia compared to women with uncomplicated

pregnancies (7.5±0.3 m/s *versus* 6.8±0.3 m/s, p=0.08, two-tailed unpaired Student's *t*-test (Figure 37)).

6.4 DISCUSSION

Pulse wave velocity and AI are measures of central and systemic arterial stiffness, which together provide a comprehensive assessment of arterial function. They are highly reproducible and have been validated in healthy subjects and those with cardiovascular disease [Chen *et al*, 1997; Fetics *et al*, 1999; Wilkinson *et al*, 2001a]. In this longitudinal study, systemic arterial stiffness decreases during the luteal phase before rising again at the beginning of the menstrual cycle. Pre-eclampsia is associated with increased arterial stiffness and, despite blood pressure returning to within the normal range, this persists in the immediate post-partum period. Increased arterial stiffness therefore appears to be a feature of pre-eclampsia that extends beyond pregnancy and may contribute to the adverse cardiovascular outcomes associated with pre-eclampsia.

6.4.1 EFFECT OF THE MENSTRUAL CYCLE

The present study is the first to use AI to determine the effect of the menstrual cycle on systemic arterial stiffness. This study demonstrates that AI is reduced in the luteal phase of the cycle indicating decreased systemic arterial stiffness. These findings differ from previous studies that demonstrate either no change [Willekes *et al*, 1997] or an increase in compliance in the ovulatory phase compared to the follicular and luteal phase [Giannattasio *et al*, 1999; Williams *et al*, 2001; Hayashi *et al*, 2006]. A

variety of factors may account for these seemingly discrepant findings, in particular, the differing methodologies employed, sample population characteristics and timing of sampling. This current study used AI as a method of evaluating systemic arterial stiffness, whereas the other studies assessed whole body arterial compliance, which combines both central and peripheral measures [Williams *et al,* 2001], or carotid artery compliance, a surrogate for aortic compliance [Hayashi *et al*, 2006]. In this current longitudinal study, clear differences exist in AI depending on the day of study. This variation will be magnified if a broader sampling time window is used as in the study by Giannattasio *et al* [Giannattasio *et al* 1999]. The study group reported in this chapter was well characterised; all women had clear evidence of ovulation as indicated by a rise in luteal phase progesterone. Despite this, neither absolute nor change in serum hormone concentrations correlated with change in AI in this study. This perhaps reflects the small numbers of women in our study. Alternatively it may imply that these hormones do not directly regulate arterial stiffness and that other intermediate factors such as the renin-angiotensin [Chidambaram *et al*, 2002] or endothelin [Polderman *et al*, 2000] systems regulate vascular tone and augmentation pressure during the menstrual cycle.

6.4.2 Effect of healthy pregnancy

Due to the logistical difficulties in obtaining pre-pregnancy data for pregnant women, pregnancy data was compared to data obtained from the same women 7 weeks post-partum. Although many cardiovascular parameters normalise rapidly over the first 2 weeks post-delivery, many require a longer time frame to settle and probably do not fully recover to pre-conceptual values [Duvekot and Peeters, 1994].

Moreover, Bernstein *et al* [2005] demonstrate that mean arterial pressure is lower in subsequent normal pregnancies than in first pregnancies and that a shorter interval between pregnancies leads to a greater reduction in mean arterial pressure. Together these studies suggest that structural vascular changes occur in pregnancy and persist beyond the gestational period [Bernstein *et al*, 2005]. The non-pregnant group of women were not used as a comparator because, although they were well matched for potential confounders including age, BMI and parity, these women were taller than the pregnant group: height being a major determinant of AI [McEniery *et al*, 2006].

The findings of a rise in central and systemic arterial stiffness from the second trimester to term and post-partum are supportive of previous studies by Oyama-Kato *et al* who used brachial-ankle PWV, a composite measure of systemic and central stiffness [Oyama-Kato *et al*, 2006]. Macedo *et al* [2008] also described this variation in their recently published cross-sectional study of AI. Other studies report no change in PWV with gestation [Rang *et al*, 2007] or a general decrease in PWV and AI over the whole of pregnancy [Poppas *et al*, 1997; Edouard *et al*, 1998; Smith *et al*, 2004; Mersich *et al*, 2005]. A variety of factors may account for these discrepant findings, but in particular the timing of sampling. All these studies had limited and wide time points with the third trimester visits performed earlier than in this study. These previous study limitations potentially explain why the subtle rise in PWV and AI in the third trimester went undetected.

The relative reduction in arterial stiffness during pregnancy compared to post-partum is likely to arise from several factors. Oestrogen has favourable effects on the

endothelium and vascular smooth muscle cells [Mendelsohn and Karas, 1999]. Indeed, many of the haemodynamic changes observed in normal pregnancy can be mimicked in non-pregnant animals through chronic exposure to oestrogen [Magness *et al*, 1993]. Both the endothelium and vascular smooth muscle cells express receptors for oestrogen and progesterone [Orshal and Khalil, 2004] through which they can regulate vascular tone [Skafar *et al*, 1997]. Therefore, they are likely to influence arterial stiffness through effects on mean arterial pressure as well as structural changes to elastin, collagen and smooth muscle in the arterial wall [Natoli et al, 2005; Payne and Webb, 2006]. Progesterone has often been thought to have opposing vascular effects to oestradiol although it has favourable vascular effects i*n vitro* [Natoli *et al*, 2005] and *in vivo* [Skafar *et al*, 1997]. However, despite the increased serum oestradiol and progesterone concentrations with advanced gestation, arterial stiffness increased in the third trimester, which may be due to mechanisms other than the action of sex steroids.

6.4.3 Effect of pre-eclampsia

Consistent with previous cross-sectional studies, all the variables of systemic and central arterial stiffness measured were higher in women with pre-eclampsia [Elvan-Taspinar *et al*, 2004; Ronnback *et al*, 2005; Spasojevic *et al*, 2005]. The possibility that medication influenced the collected data cannot be excluded because the effect of anti-hypertensive agents on PWV and AI has not specifically been studied during pregnancy. Given calcium channel blockers [Saito *et al*, 1990] and beta-blockers [Kelly *et al*, 1989] reduce PWV in non-pregnant populations, it seems likely that the

increase in arterial stiffness observed in women with pre-eclampsia, would have been even greater if these women were not taking anti-hypertensive agents.

The present studies findings contrast with a cross-sectional study by Lampinen *et al* [2006] which reported no difference in arterial stiffness assessed by AI in women with a history of pre-eclampsia. However this study was performed on average 5 to 6 years after the index pregnancy and it is possible that applanation tonometry is not sensitive enough to detect more subtle remote effects. Similarly, Spasojevic *et al* [2005] found no difference in AI between women with pre-eclampsia and healthy pregnant women at a 6-week post-partum visit. In this current study, a comprehensive assessment of arterial function demonstrated that AI and carotid-femoral PWV remained elevated at 7 weeks in women with pre-eclampsia compared to women with uncomplicated pregnancies.

Interestingly, carotid-radial PWV, unlike our other measures of arterial stiffness, had normalised by 7 weeks post-partum. Carotid-radial PWV is partly determined by the muscular brachial artery whereas carotid-femoral PWV is determined by the more elastic aorta. Carotid-radial PWV is therefore susceptible to changes in both vascular smooth muscle tone and smooth muscle remodelling. It is therefore plausible that the increase in carotid-radial PWV in pre-eclampsia and pregnancy is in part due to an effect on smooth muscle function that may normalise more rapidly post-partum than any effect on the extracellular elastin-collagen matrix of the aorta. Other conditions such as diabetes mellitus and ageing, are known to have preferential effects on central rather than peripheral arteries [Kimoto *et al*, 2003] and it is therefore perhaps

not surprising that vascular remodelling in pregnancy similarly does not occur in a uniform manner.

Carotid-femoral PWV is recognised as the gold-standard measure of arterial stiffness, as stated in the recent expert consensus document on the measurement of arterial stiffness [Laurent *et al*, 2001]. In this cohort, carotid-femoral PWV remained elevated at 7 weeks post-partum suggesting the effects of pre-eclampsia on vascular structure and function extend beyond pregnancy. If arterial stiffness remains elevated in later life, this may in part contribute to the increased risk of cardiovascular and cerebrovascular disease [Wilson *et al*, 2003].

Abnormalities of arterial structure and function were associated with higher post-partum blood pressures although these women were no longer hypertensive with blood pressures within the normal range. It is not possible from these studies to determine whether raised blood pressure during pre-eclampsia is a cause or consequence of increased arterial stiffness. There is now good evidence to suggest that aortic stiffness is an independent predictor of progression to hypertension even in young non-hypertensive individuals [Dernellis *et al*, 2005] with endothelial function being inversely related to arterial stiffness in healthy volunteers [McEniery *et al*, 2006]. Therefore, it can be postulated that in pre-eclampsia, endothelial dysfunction increases aortic stiffness, which in turn causes an increase in blood pressure.

Alternative interpretation of the data presented in this chapter is possible and it is not possible to discount that changes in arterial stiffness occur as a consequence of prolonged hypertension in pre-eclampsia. It is not known that changes in arterial stiffness at 7 weeks post-partum persist long-term. A prospective study, in which blood pressure and arterial stiffness were determined in a very large cohort of pregnant woman prior to the onset of pre-eclampsia with long-term post-partum follow-up, would be required to address these issues.

6.5 SUMMARY

The data presented in this chapter demonstrates that systemic arterial stiffness decreases during the luteal phase of the menstrual cycle with arterial stiffness rising from the mid-trimester of pregnancy to term. The factors regulating these changes in arterial stiffness have yet to be identified. However, pre-eclampsia is associated with increased arterial stiffness and this persists into the post-partum period. Increased arterial stiffness therefore appears to be a feature of pre-eclampsia that extends beyond pregnancy and suggests an abnormality of vascular structure and function associated with this condition, perhaps contributing to its adverse cardiovascular outcomes.

CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY OF FINDINGS

This thesis provides a comprehensive characterisation of markers of thrombosis, angiogenesis and arterial stiffness during the menstrual cycle, healthy and pre-eclamptic pregnancies. These studies are particularly relevant to reproductive processes where thrombotic events can have devastating outcomes and long-term vascular health may be influenced by reproductive health. The main findings and conclusions from this research are summarised below.

7.1.1 MENSTRUAL CYCLE

Using a panel of cellular and soluble markers of platelet activation, no significant variation in any of the measured markers of platelet or monocyte activation was observed within the menstrual cycle. In contrast, circulating EPC concentrations were increased in the follicular phase of the menstrual cycle suggesting a potential role for these cells in the normal cycle of physiological angiogenesis and repair of the endometrium. Finally, arterial stiffness decreases in the luteal phase of the normal menstrual cycle when both oestrogen and progesterone levels are raised. This decrease in stiffness is similar to that observed in the second trimester of healthy pregnancy and is most likely to represent a functional rather than structural change of the vasculature.

7.1.2 HEALTHY PREGNANCY

In healthy pregnancy, circulating levels of PAI-1, together with cellular and soluble markers of platelet and monocyte activation; surface P-selectin expression, platelet-monocyte aggregates, soluble P-selectin concentrations and monocyte expression of CD11b were increased during the third trimester of pregnancy. Net release of active t-PA from the endothelium was reduced in response to stimulation with bradykinin in healthy women in the third trimester of pregnancy. The impaired fibrinolytic capacity in healthy pregnancy together with the increased platelet activation may, in part, explain the increased thrombotic risk associated with pregnancy.

Consistent with an increase in soluble IL-6 and soluble ICAM-1 concentrations, CFU-EPCs were reduced during healthy pregnancy compared to non-pregnant controls. Moreover, the lack of correlation between EPCs identified by surface marker expression and those identified by CFU-EPC formation suggests that each assay potentially measures different cell types. Finally, central and systemic arterial stiffness increased over the second half of pregnancy to term despite the increased serum oestradiol and progesterone concentrations. This suggests that other mechanisms, in addition to the action of sex steroids, are involved in determining compliance in late pregnancy.

These comprehensive studies highlight the functional interplay between platelets, monocytes, EPCs and the endothelium represented by: increased markers of platelet activation and inflammation, decreased EPC-CFU formation and decreased

fibrinolytic capacity, leading to the pro-inflammatory, pro-thrombotic state of healthy pregnancy (Figure 38).

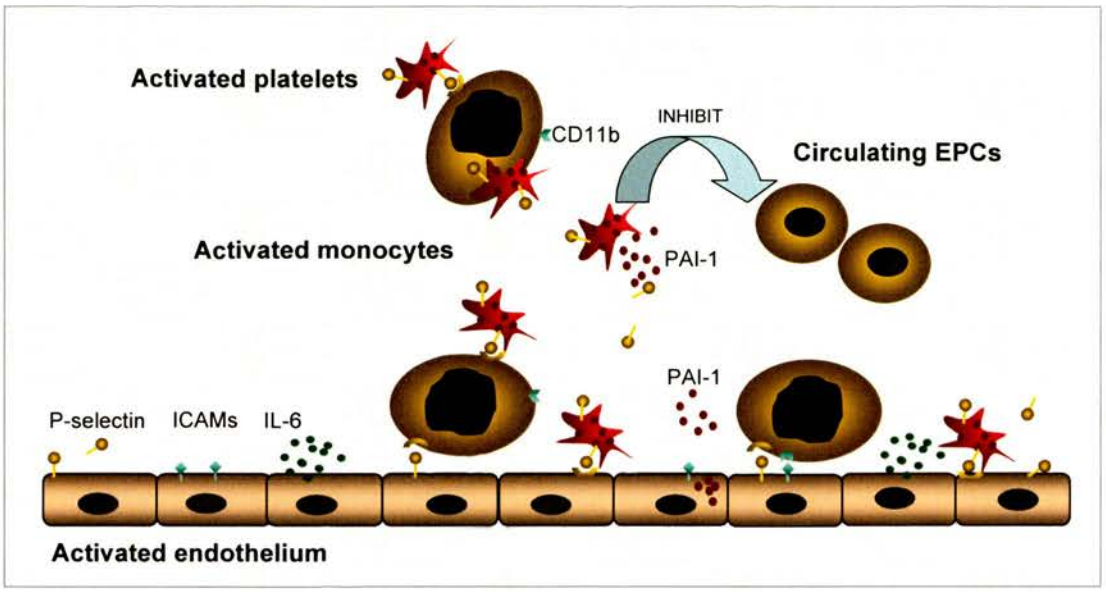

Figure 38. The functional interplay between platelets, monocytes, EPCs (endothelial progenitor cells) and the endothelium.
Platelet α-granules contain large quantities of PAI-1 (plasminogen activator inhibitor type 1), which is released upon activation. Activated platelets also activate endothelial cells by releasing IL (interleukin)-1β and monocytes, as demonstrated in our studies by increased monocyte CD (cluster of differentiation)11b. Activated monocytes themselves are pro-thrombotic, more migratory and activate endothelial cells. Activated endothelium expresses ICAMs (intercellular adhesion molecules) and releases IL-6 and PAI-1. Soluble P-selectin was measured as a marker of platelet activation, but is also shed from activated endothelium.
In vivo and *in vitro* studies suggest that activated platelets have an inhibitory effect on the proliferative and migratory capacity of EPCs from healthy volunteers [Dernbach *et al*, 2008]. This supports our finding of decreased CFU (colony forming unit) formation by mononuclear cells from healthy pregnant women, in the presence of raised circulating inflammatory markers and platelet activation.

7.1.3 PRE-ECLAMPSIA

Circulating markers of platelet activation (surface P-selectin expression, platelet-monocyte aggregates, soluble P-selectin), cytokines (IL-6 and soluble ICAM-1), circulating EPCs and CFU-EPCs were not different in women with established pre-eclampsia compared to women at similar gestational ages. However, this does not exclude the possibility that the local endothelial response to these factors within the utero-placental unit may differ in women with pre-eclampsia. Moreover, the demonstration that measures of systemic and central arterial stiffness were increased in pre-eclamptic women, persisting into the immediate post-partum period; suggests that an abnormality of vascular structure and function, may underlie the pathogenesis of this condition and its adverse cardiovascular outcomes.

7.2 DISCUSSION AND FUTURE DIRECTIONS

7.2.1 MENSTRUAL CYCLE

The normal menstrual cycle provides a state of systemic hormonal fluctuation in response to physiological activity in the ovary and uterus. Both menstruation and ovulation are considered to be inflammatory events and the cyclical regeneration of the endometrium is the largest site of physiological angiogenesis occurring in the body. It was anticipated that results from these studies would further our understanding of the temporal variation in myocardial infarction risk during the cycle and the role of circulating cells, in particular platelets and EPCs in reproductive processes.

Thrombosis

Menstruation is an inflammatory event. However, surprisingly, this work found no difference in markers of platelet activation in peripheral blood throughout the menstrual cycle. It must be acknowledged though that there was no sampling point in the late luteal phase of the cycle, just preceding menstruation. It may be that a peak in platelet activation occurs at this time, prior to the onset of menses. Future studies would investigate this and also the role of platelets in pathologies such as heavy menstrual bleeding.

Although there was no difference in markers of platelet activation within peripheral blood, the endometrium itself was not examined. Further understanding of the role of platelets in the endometrium, would include the following areas: -

1) Endometrial sampling at various points in the cycle to examine for platelets and markers of activation within the tissue itself. This could be performed either using a pipelle endometrial biopsy system or by examining menstrual blood.

2) Examination of sections of uteri obtained at hysterectomy would also potentially allow identification, localisation and determination of activation status of platelets within the endometrium.

Angiogenesis

This study lends further support to the hypothesis that circulating cells in the peripheral blood may contribute to endometrial angiogenesis. If proven, this may explain the observed failure rates after endometrial ablation techniques, where despite initial destruction or removal of all endometrial tissue, complete

amenorrhoea is rare [Lethaby *et al*, 2005]. It may also further our understanding of endometriosis, whereby endometrial tissue is found outwith the uterus. Traditionally endometriosis is thought to be caused by retrograde menstruation, through the fallopian tubes with ectopic deposition of tissue. The involvement of circulating EPCs would help explain the presence of endometrial tissue at sites distant from the peritoneal cavity, such as the lung and brain.

Further understanding of EPCs and their role in endometrial angiogenesis, would include the following areas: -

1) The use of minimally invasive pipelle endometrial sampling with histological techniques to identify EPCs within endometrial tissue of women at different stages of the menstrual cycle.

2) Targeted endometrial biopsy under direct vision (using hysteroscopy) or ultrasound guidance would potentially allow identification and localisation of EPCs within the endometrium.

3) Histological examination of sections of uteri obtained at hysterectomy would also potentially allow identification and localisation of EPCs within the endometrium. Knowledge of the menstrual cycle phase at time of hysterectomy would be valuable to further understand the role of these cells in the endometrium.

4) The potential identification of EPCs within endometriotic lesions obtained during pelvic surgery for endometriosis.

5) The measurement of circulating EPCs in the peripheral blood of women known to have endometriosis or heavy menstrual bleeding.

6) Identification of women with previous bone marrow transplants and human leukocyte antigen-mismatch and subsequent endometrial sampling or biopsy to confirm and determine the extent of involvement of bone marrow derived cells to endometrial angiogenesis.

Arterial stiffness

This research has demonstrated that the physiological changes associated with the menstrual cycle affect systemic arterial stiffness. No simple relationship was identified between the ovarian hormones and arterial stiffness. Therefore, future work would examine the relationship between other factors such as the renin-angiotensin system and circulating levels of endothelin-1 with arterial stiffness during the menstrual cycle.

7.2.2 HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Pregnancy, necessitating gross vascular adaptation accompanied by inflammation is a stressor for the vascular endothelium, similar to that experienced in sepsis [Sacks *et al*, 1998]. Overall, this research complements and adds to existing literature demonstrating that the third trimester of pregnancy is characterised by systemic inflammation, pro-thrombotic changes and a relative increase in arterial stiffness. The latter two are likely to be protective for delivery, where haemorrhage is the greatest threat to the pregnant woman. Indeed, in developing counties, haemorrhage is still the leading cause of maternal death. However, in developed countries, where haemorrhage is prevented or treated, thrombosis and thromboembolism are the leading causes and are increasing [Lewis, 2005]. In particular, in the

United Kingdom, relative and absolute numbers of deaths from myocardial infarction in pregnant and post-partum women are rising. These increases in inflammatory and thrombotic pregnancy complications are thought to be due to the current changes in women's health and the subsequent adverse clinical profile of women entering pregnancy such as increasing maternal age and obesity.

It was anticipated that this research would provide information about vascular function in the first trimester of pregnancy. Unfortunately, our earliest samples were from women in the second trimester. Women were recruited from booking clinics with the hope of getting first trimester samples. However, in these clinics the vast majority of women were greater than 12 weeks gestation. Recruiting women in their first trimester is challenging to integrate with traditional clinic design. Future studies would approach women in different settings such as early pregnancy clinics and primary care where the majority of women first present. Recruitment of such women would enable assessment of vascular function in the first trimester.

Similarly, this research provides insights into features of pre-eclampsia. Further work would measure these indicators of vascular function prospectively, in large numbers of women, in order to obtain data preceding the clinical diagnostic signs of hypertension and proteinuria, and also prior to pregnancy. In addition a longer post-natal follow-up is required to determine the longevity of our findings such as increased arterial stiffness.

Thrombosis

The third trimester of both healthy and pre-eclamptic pregnancies was associated with platelet activation. Although there was no difference in circulating cytokines or markers of platelet activation in the samples obtained from women with pre-eclampsia; the endothelium is the critical regulator of platelets, monocytes and their effects on coagulation. Endothelial cell activation is known to be heterogeneous, for example: vWF expression is greatest in the heart and lung, low in the kidney and can vary between neighbouring endothelial cells in same organ. Tissue plasminogen activator expression is greatest in the brain. In sepsis there is vascular bed-specific haemostasis and this is likely to exist in pregnancy [Rosenberg and Aird, 1999]. Therefore, although we could not detect systemic platelet activation or raised cytokine levels in women with pre-eclampsia, the endothelial response to these platelets, monocytes and cytokines may be different in women with pre-eclampsia, particularly within the utero-placental circulation. Future work would be directed to assess this, specifically by examining for the presence of platelet-monocyte aggregates in the decidua and myometrium. This would be performed by targeted placental bed biopsies obtained at time of caesarean section in women with uncomplicated pregnancies and in women with pre-eclampsia.

As discussed in chapter one of this thesis (section 1.7.5) women with pre-eclampsia are at increased risk of thrombotic events. To date, the endogenous fibrinolytic capacity of the endothelium has not been assessed. Future studies would aim to assess this using the technique of forearm venous sampling and plethysmography during intra-arterial infusion of bradykinin as was performed in Chapter 3. However

such studies are technically challenging, as is the recruitment and study of women with pre-eclampsia in the often limited time before delivery.

Angiogenesis

Our study of the role of EPCs in pregnancy was limited to those circulating in peripheral blood and as mentioned above it was difficult to obtain data for women in the first trimester of pregnancy. However, if EPCs indeed play a role in endometrial angiogenesis then it is likely that they may contribute to placentation. Isolated animal and clinical studies suggest this but further studies are required [Risau 1995; Taylor 2004**;** Bratincsak *et al*, 2007].

These would involve: -

1) Obtaining first trimester placental and decidual tissue from women undergoing surgical termination of pregnancy in order to identify the presence of these cells - using *in situ* immunohistochemistry techniques or flow cytometry.

2) Achieving pregnancies in mice following irradiation and subsequent labelled bone marrow transplantation would enable the potential identification of bone marrow derived cells within the placenta and decidua.

3) Once techniques are established for reliably identifying EPCs or more broadly, bone marrow derived cells, future work would focus on their presence in placental and decidual tissue of women with pre-eclampsia.

Arterial stiffness

This research has provided important understanding of the changes in arterial stiffness that occur in normal pregnancy. It has also demonstrated that pre-eclamptic women have increased arterial stiffness that persists beyond pregnancy. This complements a wealth of clinical studies, which report abnormal vascular function, ranging from impaired endothelium-dependent vasodilatation [Lampinen *et al*, 2006] and microvascular function to elevated VCAM, ICAM and HbA1C up to 25 years after a pregnancy complicated by pre-eclampsia [Ramsay *et al*, 2003; Sattar *et al*, 2003]. Together these data suggest that increased vascular risk may be an additive effect of pre-eclampsia to pre-pregnancy endothelial function.

Current clinical practice relies on the measurement of brachial blood pressure in pregnancy to screen for pre-eclampsia and to assess response to hypertensive therapy in women with pre-eclampsia. However, the measurement of brachial blood pressure has its challenges, increasingly so as more pregnant women are classified as obese. Inappropriate use of a blood pressure cuff that is too small can lead to over estimation of both systolic and diastolic blood pressures. As aortic compliance is a determinant of blood pressure, there maybe a future clinical role for measuring PWV instead of brachial blood pressure in some patient groups. The measurement of PWV described in this thesis requires an experienced operator and is time consuming, but alternative operator independent methods, such as the use of neck and thigh cuffs are already being used in studies of healthy volunteers.

Large-scale prospective studies are required to determine whether changes in arterial stiffness precede a rise in brachial blood pressure in pregnancies complicated by pre-eclampsia. The effect of anti-hypertensive medication upon measures of arterial stiffness in pregnancy is unknown. There is data from non-pregnant populations that suggests PWV is a significant predictor of response to anti-hypertensive therapy [Protogerou *et al*, 2009]. In women with pre-eclampsia, blood pressure control is crucial to reduce the maternal risk of stroke without compromising the utero-placental circulation. Therefore PWV may have a role in the clinical management of these women in assessing response or likelihood or response to therapy.

7.2.3 Pregnancy, pre-eclampsia and cardiovascular risk

It is known that endothelial function can be altered in childhood. Lipid parameters in childhood are strongly correlated with adult values [Raitakari *et al*, 2003]. Entering pregnancy with sub-clinical changes in endothelial function may explain the link between cardiovascular risk factors and pregnancy complications such as pre-eclampsia, gestational diabetes, hypertension, and intra-uterine growth restriction. Thus pregnancy stresses and unmasks potential endothelial problems; pregnancy complications can be considered as vascular related events, the first clinical manifestation of disruption in vascular function [Sattar and Greer, 2002]. This hypothesis may further explain the association between pregnancy complications and vascular diseases in later life, both are associated with sub-clinical changes in endothelial function and inflammation that are revealed by the added insults of pregnancy or ageing.

To test this hypothesis, a large-scale prospective study is required, examining indicators of vascular function, such as those described in this thesis and others such as high-sensitivity c-reactive protein and lipid profiles in adolescent girls prior to pregnancy. These subjects would need to be prospectively followed and further data collected on vascular function if pregnancy was achieved. Relating pre-pregnancy vascular function to the development of complications in pregnancy is vital to further understand the pathology of these conditions. Post-partum follow-up would contribute to our understanding of increased vascular risk in later life. Identifying and understanding pre-pregnancy vascular function and its subtle changes may provide the stimulus for lifestyle intervention.

Whilst the epidemiological links between pregnancy complications and increased vascular disease are widely documented there is also data to suggest that pregnancy itself increases the risk of cardiovascular disease. Epidemiological data report that multiparity is associated with increased risk of cardiovascular disease in later life, but there are multiple confounders such as socioeconomic status and psychological stress associated with child rearing. Supportive of this, men have increased risks of cardiovascular disease when fathering more than two children, though their risk is not as great as women [Lawlor *et al*, 2003]. Therefore, clinical and animal studies are likely to be more useful in understanding the sustained effects of uncomplicated pregnancy on the vasculature. Specifically, to examine the effects of pregnancy on large arteries such as the aorta, animal models are likely to be the most useful, allowing for assessment of structural changes within the arterial wall. The effects of multiparity can also be assessed more easily than in humans.

In light of an ageing maternal population and general increase in obesity, pregnancy complications are expected to increase. Unlike vascular disease in the older, non-pregnant population, preventative measures and treatment are limited in pregnancy. Treatment options for pre-eclampsia are limited to immediate anti-hypertensive therapy and delivery. Neither of these modifies the additive 7-fold risk of vascular disease in later life in these women. Therefore women who have had pre-eclampsia represent a susceptible group who should be targeted for lifestyle advice, potentially vascular risk factor screening and possible intervention.

7.3 CONCLUSION

Using recognised indicators of vascular pathology from studies outwith pregnancy, this research has demonstrated that normal healthy pregnancy is an inflammatory, and pro-thrombotic state, particularly in the third trimester. These findings may, in part, explain the temporal associations between arterial and venous thromboses and pregnancy. These systemic changes were also present in pre-eclampsia. Increasingly epidemiological and clinical studies are highlighting the links between vascular disease, particularly atherosclerosis and pre-eclampsia. Further understanding the effect of pre-pregnancy vascular function and pregnancy complications could lead to targeted lifestyle modifications, screening and potentially intervention. This research also demonstrated that increased arterial stiffness was a feature of pre-eclampsia that extended beyond pregnancy. This suggests an abnormality of vascular structure and function that may underlie the pathogenesis of this condition and may contribute to

the increased immediate and lifetime risk of vascular disease in these women. Detection of persisting increased arterial stiffness, may allow for targeted treatments in these women to modify their risks of vascular disease.

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APPENDIX 1

MATERIALS AND SUPPLEMENTARY INFORMATION ON METHODS

ARTERIAL STIFFNESS STUDIES	SOURCE
Micro life 3BTO-A automated sphygmomanometer	Micro life AG, Widnau, Switzerland
Micromanometer	Millar Instruments, Texas, USA
SphygmoCor system	AtCor Medical, Sydney, Australia
VENOUS OCCLUSION PLETHYSMOGRAPHY	**SOURCE**
Semi-automated noninvasive oscillometric sphygmomanometer	OMRON 705 IT, Kyoto, Japan
Mercury-in-silastic strain gauges	Hokanson, Bellevue, WA 98005 USA
Plethysmograph	Hokanson, Bellevue, WA 98005 USA
Bradykinin	Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland.
Sodium nitroprusside	Hospira, Illinois, USA
Noradrenaline	Hospira, Illinois, USA
B-9340	Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland.
VENOUS BLOOD COLLECTION	**SOURCE**
19-Gauge needle, S-Monovette®	Sarstedt AG & Co. Nümbrecht Germany
PPACK Anticoagulant tubes (75 μM)	Cambridge BioScience, Cambridge, UK.
EDTA tubes, S-Monovette®	Sarstedt AG & Co. Nümbrecht Germany
Sodium Citrate tubes, S-Monovette®	Sarstedt AG & Co. Nümbrecht Germany
Stabilyte tubes (Acidified Bufferred Citrate)	Biopool International, Ventura, USA.
Serum Gel tubes, S-Monovette®	Sarstedt AG & Co. Nümbrecht Germany
FLOW CYTOMETRIC ANALYSIS	**SOURCE**
MOUSE ANTI-HUMAN CD42a-FITC (GRP-P, IgG1)	AbD Serotec Ltd, Oxford, UK.
MOUSE 1gG1 NEGATIVE CONTROL:FITC	AbD Serotec Ltd, Oxford, UK.
MOUSE ANTI-HUMAN CD14-FITC	AbD Serotec Ltd, Oxford, UK.
MOUSE ANTI-HUMAN CD40-RPE	AbD Serotec Ltd, Oxford, UK

MOUSE ANTI-HUMAN CD11b-RPE	AbD Serotec Ltd, Oxford, UK
MOUSE ANTI-HUMAN CD14-PE (Tuk-4, IgG2a)	DakoCytomation UK Ltd, Ely, UK.
MOUSE IgG2a NEGATIVE CONTROL:PHYCOERYTHRIN	DakoCytomation UK Ltd, Ely, UK.
MOUSE ANTI-HUMAN CD62P-PE (IgG2a)	DakoCytomation UK Ltd, Ely, UK.
MOUSE IgG1 NEGATIVE CONTROL: PE	DakoCytomation UK Ltd, Ely, UK.
MOUSE ANTI-HUMAN CD34-FITC	Becton Dickinson UK Ltd, Oxford, UK
MOUSE ANTI-HUMAN CD133-PE	Miltenyi Biotec Ltd, Bisley, UK
Mouse monoclonal anti-humanVEGF R2 (KDR)-APC	R&D Systems Europe Ltd, Abingdon, UK
Mouse IgG1 Isotype Control-APC	R&D Systems Europe Ltd, Abingdon, UK
Uti-Lyse™ Erythrocyte Lysing Reagent	DakoCytomation UK Ltd, Ely, UK.
Phosphate buffered saline (PBS) without cations	Sigma-Aldrich, Poole, UK
FACS-Lysing Solution (See Appendix 2)	Becton Dickinson UK Ltd, Oxford, UK
Flow Buffer	See Appendix 2
Anti-Mouse Ig,κ/Negative Control Compensation Particles Set	BD Biosciences, Oxford, UK
BD FACSCalibur Flow Cytometer	BD Biosciences, Oxford, UK
Flowjo software	Tree Star, Inc. Oregon, USA
***IN VITRO* CULTURE**	**SOURCE**
6-well and 24-well fibronectin-coated plates	Becton Dickinson UK Ltd, Oxford, UK
Histopaque	Sigma-Aldrich Company Ltd, Poole, UK
EndoCult™ Liquid Medium Kit	StemCell Technologies, Grenoble, France
PenStrep, (containing penicillin and streptomycin),	StemCell Technologies, Grenoble, France
Trypan Blue	Sigma-Aldrich Company Ltd, Poole, UK
Endothelium Growth Medium-2	Lonza Ltd, Basel, Switzerland.

IMMUNOFLUORESENCE	**SOURCE**
Fibronectin-coated chamber slides	Becton Dickinson UK Ltd, Oxford, UK
Dil-AcLDL (1,1'-dioctadecyl-3,3,3',3' tetramethylcarbocyanine-labelled acetylated LDL)	Biomedical Technologies Inc, MA, USA.
FITC-labelled Lectin from *Ulex europaeus*	Sigma-Aldrich Company Ltd, Poole, UK
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich Company Ltd, Poole, UK
Permafluor	Immunotech, High Wycombe, UK
10% goat serum	Autogen Bioclear, Calne, UK
1% BOVINE SERUM ALBUMIN	Sigma-Aldrich Company Ltd, Poole, UK
0.02% IGEPAL/NP40	Sigma-Aldrich Company Ltd, Poole, UK
Primary murine monoclonal antibodies against human CD105	Becton Dickinson UK Ltd, Oxford, UK
Primary murine monoclonal antibodies against human CD146	Springbioscience, Heidleberg, Germany
Polyclonal goat anti-mouse biotinylated antibody	DakoCytomation UK Ltd, Ely, UK
Streptavidin-Alexa Fluor 546	Molecular probes®, Invitrogen, California, USA
Streptavidin-Alexa Fluor 488	Molecular probes®, Invitrogen, California, USA
Human Umbilical Vein Endothelial Cells	Dr C Shaw, Department of Cardiovascular Research, University of Edinburgh
Human colon cell-line (HT29)	Dr S Bader, Edinburgh Cancer Research Centre
Zeiss 'LSM 510 Meta' confocal microscope	Carl Zeiss, Oberkochen, Germany
ELISAs	**SOURCE**
Oestradiol ELISA	Siemens Medical Solutions Centaur, Camberley, UK
Progesterone ELISA	Siemens Medical Solutions Centaur, Camberley, UK
Lutenising Hormone ELISA	Abbott Architect, Illinois, USA

Follicular-Stimulating Hormone ELISA	Abbott Architect, Illinois, USA
Soluble P-Selectin ELISA	R&D Systems Europe Ltd, Abingdon, UK
CD40 Ligand ELISA	R&D Systems Europe Ltd, Abingdon, UK
IL-6 ELISA	R&D Systems Europe Ltd, Abingdon, UK
Soluble ICAM-1 ELISA	R&D Systems Europe Ltd, Abingdon, UK
VEGF-ELISA	Calbiochem, Darmstadt, Germany
t-PA ELISA, t-PA Combi Actibind Elisa Kit	Technoclone, Vienna, Austria
PAI-1 ELISA, Elitest PAI-1 Antigen and Zymutest PAI-1 Activity	Hyphen Biomed, Neuville-Sur-Oise, France
STATISTICAL ANALYSIS	**SOURCE**
Graph Pad Prism	(GraphPad Software, USA)

APPENDIX 2

RECIPES FOR SOLUTIONS

ALL DILUTIONS ARE IN DEIONIZED WATER, UNLESS OTHERWISE STATED

Bradykinin (for in vivo use within venous occlusion plethysmography studies, Chapter 3)

1 vial contains: 69 μg of bradykinin

Batch No: AC 0556 Molecular weight: 1060.2

Reconstitution of 1 vial using 0.9% NaCl:

1 vial of 69 μg in 65 mL 0.9% saline

= 1000 pmol/mL

Add 15 mL of 1000 pmol/mL to 35 mL of 0.9% saline (total vol 50 mL)

= 300 pmol/mL

Add 15 mL of 300 pmol/mL to 30 mL 0.9% saline (total vol 45 mL)

= 100 pmol/mL

Sodium nitroprusside (SNP, for in vivo use within venous occlusion plethysmography studies – Chapter 3)

1 vial contains: 50 mg SNP (dry powder)

Reconstitution of 1 vial using 09.% NaCl:

50 mg SNP diluted into 500 mL saline = 100 μg/mL

(use 2 mL of saline from 500 mL bag to dilute powder)

Add 4 mL of 100 μg/mL to 46mL saline (total volume of 50 mL)

= 8 μg/mL

Add 20 mL of 8 μg/mL to 20 mL saline (total volume of 40 mL)

= 4 μg/mL

Add 20 mL of 4 μg/mL to 20 mL saline (total volume of 40 mL)

= 2 μg/mL

Flow Buffer (for platelet flow cytometric studies,Chapter 4)

Phosphate buffered saline without cations

0.1% Bovine Serum Albumin

0.1% Sodium Azide

1% Paraformaldehyde

EndoCult™ Complete Culture Medium (ECCM, for CFU-EPC assay, Chapter 5)

2 mL EndoCult™ Supplement

8 mL EndoCult™ Basal Medium

Penicillin and Streptomycin Solution added to give final concentration of 100 U/mL of Penicillin and 100 μg/mL of Streptomycin.

Blocking Serum (for immunofluorescence of CFUs,Chapter 5)

10 mL Normal Goat Serum

80 mL Phosphate Buffered Saline

1 g Bovine Serum Albumin

0.02% IGEPAL/NP 40

APPENDIX 3

STAINING PROTOCOL FOR PLATELET-MONOCYTE AGGREGATES, PLATELET P-SELECTIN EXPRESSION, MONOCYTE CD40 AND CD11B EXPRESSION AND ENDOTHELIAL PROGENITOR CELLS

REQUIREMENTS (PER STUDY)

- 12 eppendorf tubes
- Flow buffer (phosphate buffered saline without cations with 0.1% bovine serum albumin and 0.1% sodium azide).
- FACS-lyse solution (Becton Dickinson)
- 1% paraformaldehyde
- Monoclonal antibodies (diluted with flow buffer):
    - IgG1-FITC / CD14-PE 1 :20
    - CD14-PE / CD42a-FITC
    - IgG2a-PE / CD42a-FITC
    - CD42a-FITC / CD62P-PE
    - CD42a-FITC / CD154-PE
    - CD14-FITC / CD40-PE
    - CD14-FITC / CD11b-PE

1. ***Take blood with 19-gauge or larger needle***
    a. If drawing blood from a cannula try to use minimal tourniquet pressure during insertion.
    b. Blood should come back freely, and with no resistance to avoid artefactual platelet activation. If blood is sluggish or stop-start consider trying again.

2. ***Put 3 mL into PPACK tube and start timer to count down 5 minutes.***

3. ***After exactly 5 minutess start staining:***

i. Stain blood for Platelet-monocyte aggregates (PMA)

Sample	Vol. of whole blood	Antibody type & vol	Additional reagent	Total volume
Blank	60µL	None	60µL flow buffer	120µL
CD14:RPE / IgG1-FITC	60µL	60µl IgG FITC/ CD14 1:20	none	120µL
CD14:RPE / CD42a:FITC	60µl	60µl CD14/42a 1:20	none	120µL

Set timer to count down from 20 minutes:

ii. Stain blood for P-selectin

Sample	Vol. of whole blood	Antibody type & vol	Additional reagent	Total volume
Blank	5µL	None	45µL flow buffer	50µL
IgG2a:RPE / CD42a-FITC	5µL	45µL IgG2a RPE/ CD42a FITC 1:20	none	50µL
CD62P:RPE / CD42a:FITC	5µL	45µL CD62P/CD42a 1:20	none	50µL

iii. Stain blood for Monocyte CD40 and CD11b

Sample	Vol. of whole blood	Antibody type & vol	Additional reagent	Total volume
Blank	60µL	None	60µL flow buffer	120µL
CD14:FITC (no isotype control)	60µL	60µL CD14 1:20	none	120µL
CD40:RPE / CD14:FITC	60µL	60µL CD40/CD14 1:20	none	120µL
CD11b:RPE / CD14:FITC	60µL	60µL CD11b/CD14 1:20	none	120µL

4. ***When timer reaches zero (i.e. 20 mins after staining for PMA) FIX cells as below:***

   i. Set timer for 5 minutes then add 500 µL FACS-lyse to all PMB tubes

   ii. When timer reaches zero reset for 5 minutes then add 1450 µL paraformaldehyde to all P-selectin tubes

   iii. When timer reaches zero add 500 µl FACS-lyse to all Mono tubes

How to make FACS-lyse

Add 5 mL of neat FACS-lyse to 50 mL Falcon tube. Make up to 50 mL total with Deionized or Millipore water.

- total solution 50 mL of 1:10 FACS-lyse
- will last for 1 month kept in fridge

Antibodies required

Serotec

- **MCA928F** Mouse IgG1-FITC negative control
- **MCA1227F** Mouse anti-human CD42a-FITC
- **MCA596F** Mouse anti-human CD14-FITC
- **MCA1590PE** Mouse anti-human CD40-PE
- **MCA551PE** Mouse anti-human CD11b-PE

DakoCytomation

- **R0864** Mouse anti-human CD14-PE
- **X0950** Mouse IgG2a-PE negative control
- **R7200** Mouse anti-human P-selectin CD62P-PE
- **X0928** Mouse IgG1-PE

All antibody mixes are diluted 1:20 with flow buffer. Add 50 µL of each antibody neat and make it up to 1000 µL with flow buffer for the 1:20 dilution.

For example for the platelet-monocyte panel:

50 µL IgG1-FITC [MCA928F] + 50 µL CD14-PE [R0864] + 900 µL flow buffer = 1000 µL of IgG1-FITC / CD14-PE 1:20

50 µL CD14-PE [R0864] + 50 µL CD42a-FITC [MCA1227F] + 900 µL flow buffer = 1000 µL of CD14-PE / CD42a-FITC 1:20

For the platelet P-selectin panel:

50 µL IgG2a-PE [X0950] + 50 µL CD42a-FITC [MCA1227F] + 900 µL flow buffer = 1000 µL of IgG2a-PE / CD42a-FITC 1:20

50 µL CD42a-FITC [MCA1227F] + 50 µL CD62P-PE [R7200] + 900 µL flow buffer = 1000 µl of CD42a-FITC/ CD62P-PE 1:20

For the Monocyte CD40 panel:

50 µL CD14-FITC [MCA596F] + 950 µL flow buffer = 1000 µl of CD14-FITC 1:20

50 µL CD14-FITC [MCA596F] + 50 µL CD40-PE [MCA1590PE] + 900 µL flow buffer = 1000 µL of CD14-FITC/ CD40-PE 1:20

50 µL CD14-FITC [MCA596F] + 50 µL CD11b-PE [MCA551PE] + 900 µL flow buffer = 1000 µL of CD14-FITC/ CD11b-PE 1:20

This amount of stock will be enough for 16 visits.

Flow cytometer set up

For platelet monocyte aggregates, use PMB template with PMB settings. Collect 2500 monocytes.

For P-selectin use plt template and plt settings. Collect 7500 platelets.

For Monocyte CD40 use Mono template and settings. Collect 3000 monocytes positive for CD14. There is no isotype control for this panel so please make sure you collect 3000 monocytes from the unstained sample as well - we will use this as a control.

Although the templates should be fine, all lasers and flow cytometers differ slightly and the antibody batches you get may be different to ours. Therefore, the instrument settings and compensation may need to be adjusted for your flow cytometer.

Adjusting the compensation and instrument settings should only need to be done once at the beginning when setting up the protocol. Once they are set and saved you should not have to change them again.

If you are not sure about the compensation it is much better to under-compensate or not compensate at all. Post-collection compensation can be achieved but is not possible if the data is over compensated.

Flow cytometric assay for the quantification of CD133/CD34/KDR progenitor cells in whole peripheral blood

Requirements

6 BD Falcon tubes with lids and labelled 1 to 6

PBS (without cations)

UTI-lyse reagent (from DAKO) A and B

Monoclonal antibodies:

- Mouse anti-human CD34 (Becton Dickson; FITC conjugated) used neat
- Mouse anti-human CD133 (Miltenyl Biotech; PE conjugated) used neat
- Mouse anti-human CD14 (Becton Dickson ; APC conjugated) used at 1:20 dilution
- Mouse anti-human IgG1 (R&D; APC conjugated) used neat
- Mouse anti-human KDR (R&D; APC conjugated) used neat

PROTOCOL

1. Pipette 200 μL of whole blood (from EDTA tube) into each of the 8 Falcon tubes. (Use a P1000 pipette). Send the remainder of the blood in the EDTA tube to haematology for full blood count.
2. Add antibodies as follows: - pipette up and down 3 times and then gently vortex tube.

Tube number		Antibodies to be added
1	Blank	None
2	CD34	5μL CD34
3	CD34 CD133	5μL CD34 5μL CD133
4	CD34 CD133 ISO	5μL CD34 5μL CD133 5μL ISOTYPE
5	CD34 CD133 KDR	5μL CD34 5μL CD133 5μL KDR
6	CD14	5μl CD14 (1 IN 20 DILUTION)

3. Incubate in the dark for 20 minutes.
4. At 20 minutes add 200 μL of UTI-lyse reagent A to each tube. Vortex gently. Leave for 10 minutes at room temperature in the dark.
5. Stop reaction A at 10 minutes by adding 2 mL of UTI-lyse reagent B to each tube - put numbered lids on at this stage and vortex gently. Leave for a minimum of 10 minutes in the dark.
6. Centrifuge for 5 minutes, 1500 g at 20°C.
7. Tip off supernatant onto paper towel - be careful not to cross contaminate the tops of tubes.
8. Add 1 mL of PBS (from fridge) to each tube. Replace numbered lids and vortex gently.
9. Centrifuge for 5 minutes, 1500 g at 20°C.
10. Tip off supernatant onto paper towel - be careful not to cross contaminate the tops of tubes.
11. Add 500 μL of PBS to each tube. No need to replace lids - they can be discarded.

Samples are now ready to run through the flow cytometer.

Store in the dark at room temperature until used.

PUBLICATIONS

PUBLICATIONS

Robb AO, Mills NL, Newby DE, Denison FC. Endothelial progenitor cells in pregnancy. *Reproduction* 2007;**133**(1):1-9.

Robb AO, Mills NL, Din JN *et al.* Acute endothelial tissue plasminogen activator release in pregnancy. *J Thromb Haemost* 2009;**7** (1):138-142.

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Robb AO, Mills NL, Smith IBJ *et al.* Influence of menstrual cycle on circulating endothelial progenitor cells. *Hum Reprod* 2009;**24** (3):619-625.

REPRODUCTION

REVIEW

Endothelial progenitor cells in pregnancy

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Abstract

The discovery of endothelial progenitor cells has generated considerable interest in the field of vascular biology. These cells arise from a population of circulating mononuclear cells and have the capacity to form new blood vessels and contribute to vascular repair. Circulating endothelial progenitor cell numbers are reduced in patients with cardiovascular risk factors and in the presence of endothelial dysfunction, but are increased in response to ischaemia, oestrogens and drug therapy. They have been studied in pathologies from cardiovascular and renal disease to rheumatoid arthritis and pre-eclampsia. Pregnancy is a challenge to the maternal vascular system, requiring systemic adaptation and pronounced local changes in the uterus. Diseases of pregnancy such as pre-eclampsia and gestational diabetes increase the risk of pregnancy complications and are associated with endothelial dysfunction. We propose that endothelial progenitor cells have an important role in the regulation and maintenance of the vasculature during pregnancy. This review summarises our current understanding of endothelial progenitor cells, with specific reference to their role in angiogenesis and human pregnancy.

Reproduction (2007) **133** 1–9

Introduction

The observation of an affinity between endothelial cells and haematopoietic cells was first reported in 1920 by Florence Sabin. Until recently, differentiation of endothelial cells from angioblasts was thought to exclusively occur within the developing embryo. In 1997, Asahara and colleagues isolated a population of bone marrow-derived cells from human peripheral blood capable of *ex vivo* expansion, differentiation into a mature endothelial phenotype and neovascularisation in response to acute tissue ischaemia (Asahara *et al.* 1997). Shortly after this, Shi and colleagues observed that bone marrow cells were important for the endothelialisation of intra-aortic Dacron grafts in dogs (Shi *et al.* 1998). These studies suggest the existence of a population of circulating endothelial progenitor cells (EPCs), which are mononuclear, cells arising in bone marrow with the capacity to differentiate into mature endothelial cells.

Normal human pregnancy involves adaptation of maternal vasculature to accommodate and sustain the developing foetus. Abnormal adaptation of the uterine vasculature is associated with deficient placentation and some diseases of pregnancy including pre-eclampsia and gestational diabetes are associated with systemic endothelial dysfunction (Brosens *et al.* 1972, McCarthy *et al.* 1993, Knock *et al.* 1997). We postulate that EPCs play an important role in development, regulation and maintenance of the vasculature during pregnancy. This review summarises our current understanding of the origin and function of EPCs, and highlights their potential role in angiogenesis and vascular repair in human pregnancy.

Characterisation of circulating EPCs

EPCs are characterised by their expression of both haematopoietic and mature endothelial cell antigens, and by their ability to proliferate, migrate and differentiate into mature cell types. Asahara and colleagues exploited two antigens shared by endothelial cells and haematopoietic stem cells (HSCs) to isolate putative EPCs from peripheral blood (Asahara *et al.* 1997). CD34 is expressed by most mature endothelial cells (Fina *et al.* 1990) as well as all HSCs but is lost by haematopoietic cells as they differentiate (Civin *et al.* 1984). Kinase insert domain receptor (KDR), the extracellular domain of vascular endothelial growth factor receptor (VEGFR) (Shalaby *et al.* 1995), is also expressed by both early HSCs and endothelial cells but is lost on haematopoietic cell differentiation (Matthews *et al.* 1991). $CD34^+$ and KDR^+ cells, isolated from peripheral blood leucocytes

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form vascular structures *in vitro* and incorporate into the vessel wall in experimental models of neovascularisation (Asahara *et al.* 1997).

Co-expression of CD34 and KDR has been used in a number of experimental and clinical studies to identify circulating EPCs. No surface marker unique to endothelial progenitors has been identified and so it remains difficult to distinguish EPCs from mature endothelial cells that have been swept into the circulation or haematopoietic cells. CD133 is expressed by haematopoietic cells, but not by mature endothelial cells. Identification of CD133, KDR and CD34 co-expression may differentiate between circulating mature and progenitor endothelial cells (Peichev *et al.* 2000). The rarity of EPCs in peripheral blood (100–200 cells/ml), has made their study difficult.

Alternative methods have been described for the characterisation and quantification of EPCs based on the culture of endothelial cells from circulating mononuclear cells. A number of functional assays have been reported, most involving the isolation of peripheral blood mononuclear cells by density centrifugation of blood and subsequent culture on fibronectin coated plates. After 5–7 days in culture, adherent colonies are seen, where spindle shaped cells emerge from a cluster of round cells (EPC colony forming units, EPC-CFUs). These adherent cells display a variety of endothelial-like properties including the uptake of acetylated low density lipoprotein (AcLDL) and staining with UEA-1 (Fig. 1), a lectin of *Ulex europaeus,* specific for endothelial cells in a variety of tissues binding to the carbohydrate moiety α-l-fucose (Stephenson *et al.* 1986).

Whilst counting EPC-CFUs measures the capacity of circulating mononuclear cells to form endothelial cells, the colonies may not directly arise from the $CD34^+$ stem cells. The exact phenotype of EPC-CFUs remains a matter of debate in part because the purity of $CD34^+$ cells used in the initial Asahara study was only 15% (Asahara *et al.* 1997). Peripheral blood contains several cell types that can differentiate into endothelial-like cells *in vitro,* including haematopoietic stem cells, mononuclear phagocytes (monocyte-macrophages), and mature endothelial cells (Ingram *et al.* 2005). In a recent methodological paper by George and colleagues no correlation was found between the number of peripheral blood $CD34^+$ cells and the number of EPC-CFUs (George *et al.* 2006).

Studies addressing the origin of EPCs have demonstrated that monocytes express endothelial lineage markers such as KDR and can differentiate into endothelial cells (Schmeisser *et al.* 2001). Rehman and colleagues found that the majority of EPC-CFUs expressed monocyte markers such as CD14, Mac-1, and CD11c, suggesting that peripheral blood EPCs are derived from monocyte-like cells (Rehman *et al.* 2003). The concept that functional endothelial cells may originate from a $CD14^+$ progenitor is supported by

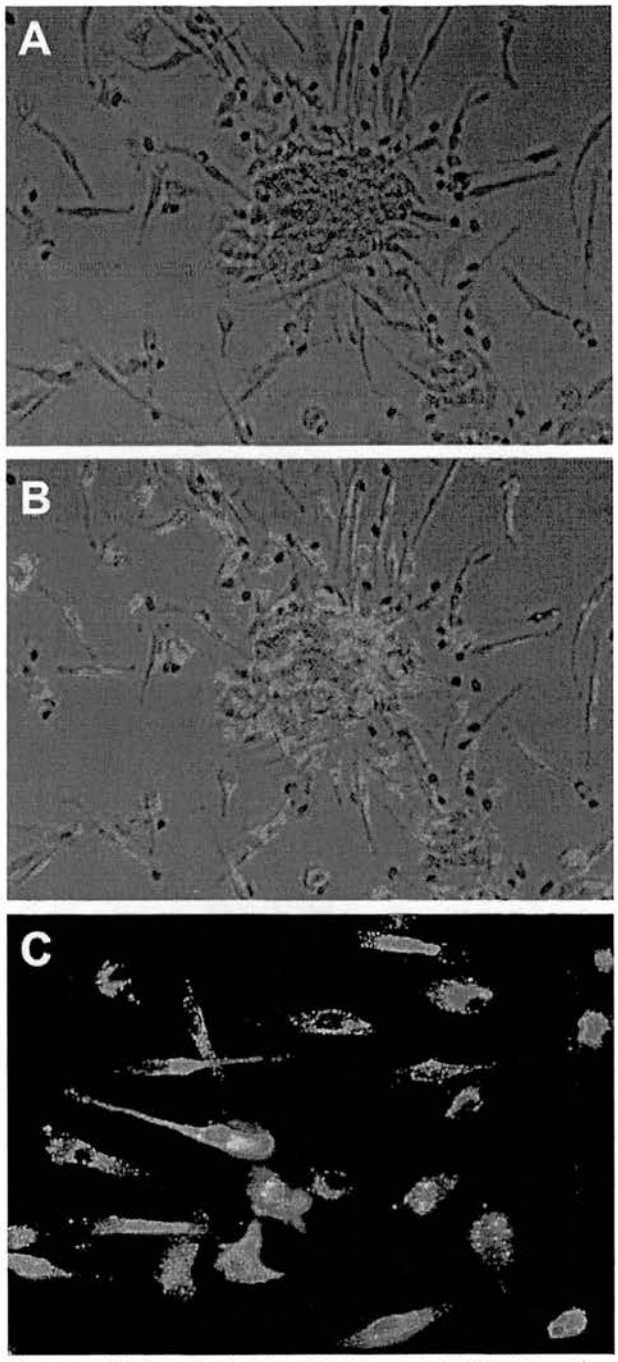

Figure 1 (A) A typical colony-forming unit (EPC-CFU) with a characteristic core of round cells and sprouting spindle cells at the periphery (× 100 magnification). (B) Overlay with immunofluorescence staining of EPC-CFU demonstrating uptake of Dil-acetylated-LDL (red) (× 100 magnification). (C) Fluorescent micrograph of cultured EPCs 4 days after isolation from peripheral blood, stained with lectin *Ulex europaeus*-FITC (green) and demonstrating uptake of Dil-acetylated-LDL (red) (× 40 magnification). Figure 1(C) reproduced with permission from Kalka *et al.* (2000).

reports that mature endothelial cells from human umbilical vein express CD14 (Jersmann *et al.* 2001) and that isolated $CD14^+$ cells can improve neovascularisation after mouse hind limb ischaemia (Urbich *et al.* 2003). We present a diagram outlining two potential ways that endothelial cells might arise from haematopoietic stem cells via myeloid or endothelial progenitor subtypes (Fig. 2).

In this emerging field, many different methods have been used to characterise and quantify putative endothelial progenitors, preventing straightforward comparison. Reduced levels of both phenotypic (Schmidt-Lucke *et al.* 2005) and functional EPCs (Werner *et al.* 2005) predict adverse outcome in patients with coronary artery disease. Whilst questions remain as to the origin and phenotype of EPCs, evidence that they contribute to vascular repair and neoangiogenesis in animal models is compelling (Table 1).

Mobilization and differentiation of EPCs

The vascular endothelium is a monolayer of cells central to the regulation of blood vessel tone and permeability. It acts as a non-adhesive surface for leucocytes and platelets, and produces important factors in the regulation of fibrinolysis and blood flow. Endothelial dysfunction caused by mechanical or biochemical stress and inflammation is characterised by reduced nitric oxide bioavailability and a progressive loss of endothelial cells (Libby 2002, Wassmann & Nickenig 2003). Endothelial denudation is one of the earliest pathophysiological features of vascular disease, with persistent endothelial dysfunction responsible for the progression and clinical manifestations of atherothrombosis (Ross 1999, Weissberg & Bennett 1999). Integrity of the vascular endothelium is a dynamic equilibrium between endothelial degeneration and repair (Karter *et al.* 2004).

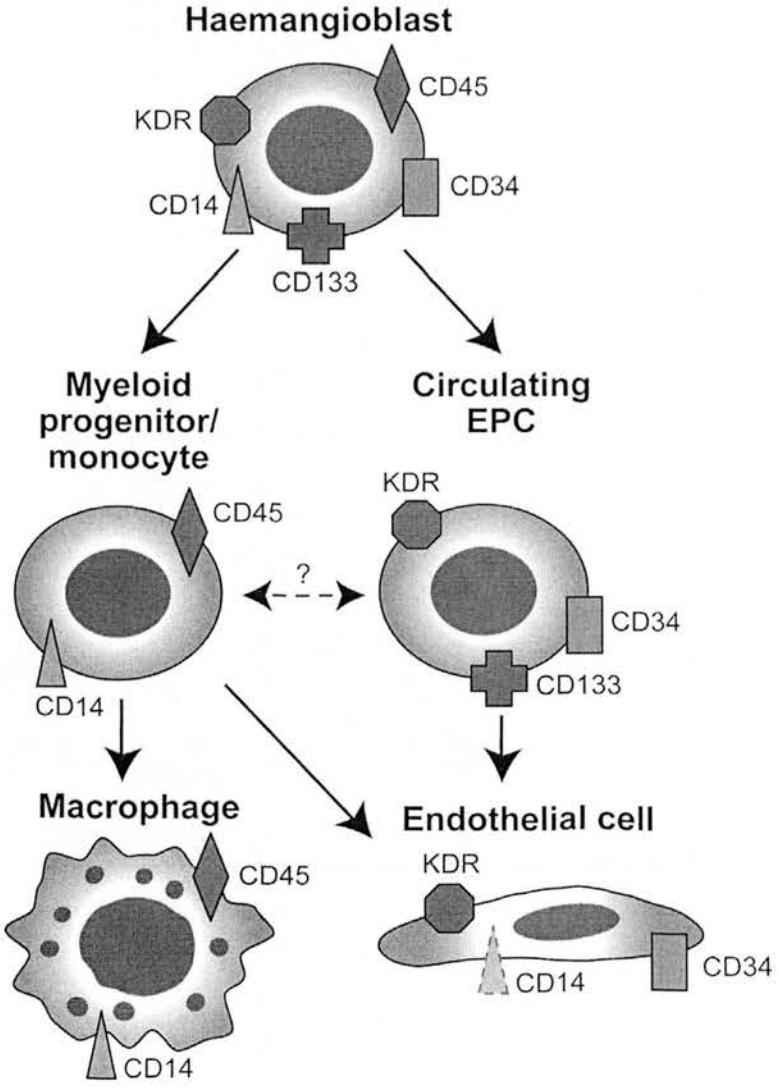

Figure 2 Possible pathways by which endothelial cells may arise from haematopoietic stem cells. Haemangioblasts give rise to circulating haematopoetic stem cells of myeloid or endothelial progenitor subtypes, with endothelial cells potentially derived from either pathway.

Table 1 Characteristics of endothelial progenitor cells

Phenotypic	Cell-surface expression of haematopoietic stem-cell (CD34, CD133) and endothelial-cell antigens (KDR)
Functional	Produce characteristic colonies and tubular structures on fibronectin *in vitro*
	Migrate and incorporate into areas of vascular damage *in vivo*
	Differentiate into mature endothelial cells

The concept of a pool of endothelial progenitors in the bone-marrow, capable of moving to effect angiogenesis or vascular repair in response to ischaemia or vascular injury is supported by both *in vitro* and *in vivo* studies. In a mouse model, Asahara and colleagues demonstrated differentiation of donor bone marrow cells into endothelial cells and their subsequent incorporation into the vasculature during processes such as ovulation, wound healing, recovery from hind limb ischaemia and neoplasia (Asahara *et al.* 1999).

Reduced circulating EPC levels are observed when established cardiovascular risk factors are present suggesting a role for EPCs in the maintenance of endothelial function. Decreased numbers of EPCs have been demonstrated in cigarette smokers (Vasa *et al.* 2001) and in patients with diabetes mellitus (Tepper *et al.* 2002) or rheumatoid arthritis (Grisar *et al.* 2005). Hill *et al.* (2003) observed a strong correlation between the Framingham cardiovascular risk score (which uses cardiovascular risk factors to predict future risk of coronary artery disease (Wilson *et al.* 1987)) and circulating EPC numbers. EPC numbers predicted systemic endothelial function more accurately than the Framingham risk score (Hill *et al.* 2003). Furthermore, lower levels of EPCs are associated with adverse outcome in patients with coronary artery disease (Schmidt-Lucke *et al.* 2005, Werner *et al.* 2005) and impaired myocardial remodelling after infarction (Leone *et al.* 2005).

The postulated factors responsible for mobilization of EPCs from the bone marrow are the subject of an intense search. Such a factor might form a therapeutic strategy to enhance vascular repair. EPCs are released in the context of acute ischaemic injury, such as myocardial infarction (Leone *et al.* 2005, Massa *et al.* 2005) and following vascular injury as a consequence of coronary artery bypass grafting (Gill *et al.* 2001). Vascular endothelial growth factor (VEGF) and stromal cell–derived factor-1 (SDF-1), both released from ischaemic tissue, are thought to be important factors in the mobilisation of EPCs. Other cytokines such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) mobilise both haematological progenitors and EPCs. These have been used for many years to harvest progenitors for autologous bone marrow transplantation in the context of haematological malignancy. However, they also induce a pro-inflammatory state which may limit their therapeutic potential.

Mechanisms of EPC mobilisation in conditions other than ischaemia may be more relevant to understanding the role of EPCs in maternal circulation during pregnancy. The effect of oestrogens in maintaining endothelial function may be related to EPC mobilisation and enhanced vascular repair. In a rat-carotid injury model, exogenous oestradiol accelerates re-endothelialisation and attenuates medial thickening via mobilization and proliferation of bone marrow-derived EPCs. This response was absent in endothelial nitric oxide synthase (eNOS) knock out animals (Iwakura *et al.* 2003). Recent mouse studies with oestrogen receptor (ER) α and β knockout animals demonstrate roles for both receptors α and β in EPC-mediated neovascularisation in response to ischaemia. In addition, ERα messenger RNA expression was higher than ERβ messenger RNA expression in EPCs. VEGF expression was significantly down-regulated on EPCs from ERα knockout mice compared with EPCs from wild type animals (Hamada *et al.* 2006).

Vascular remodelling in pregnancy

Growth of endometrial vasculature in preparation for implantation begins in the proliferative phase and continues in the secretory phase of the menstrual cycle. This physiological angiogenesis is thought to occur primarily through elongation and intussusception of existing small vessels (Gargett & Rogers 2001). Sprouting is important in placentation and is also a feature of angiogenesis associated with pathology such as ischaemia (Reynolds & Redmer 2001) (Fig. 3). A role for EPCs in physiological endometrial angiogenesis has been implicated by Asahara and colleagues where EPCs were demonstrated within vasculature and stroma of the endometrium and myometrium after induced ovulation in mice (Asahara *et al.* 1999). Increased circulating levels of EPCs are seen during the secretory phase of the menstrual cycle in human subjects (Matsubara *et al.* 2006).

The uterine vasculature undergoes dramatic remodelling during pregnancy. In addition to vasodilation of the uterine artery, remodelling of maternal spiral arteries provides a large vascular bed perfusing the placental intervillous space with maternal blood (Brosens *et al.* 1967). During placentation, this remodelling is mediated by interstitial and endovascular trophoblast invading maternal vessels (for a review see (Pijnenborg *et al.* 2006)). During the wave of trophoblast invasion, maternal spiral artery endothelium is extensively damaged and then repaired. This results in a fresh layer of endothelium (Pijnenborg *et al.* 2006). The repair is thought to be effected by local endothelial cells though it is plausible that circulating EPCs are involved.

Vascular and endothelial function in pregnancy

Pregnancy necessitates cardiovascular adaptation to sustain the developing foetus. Initially, peripheral

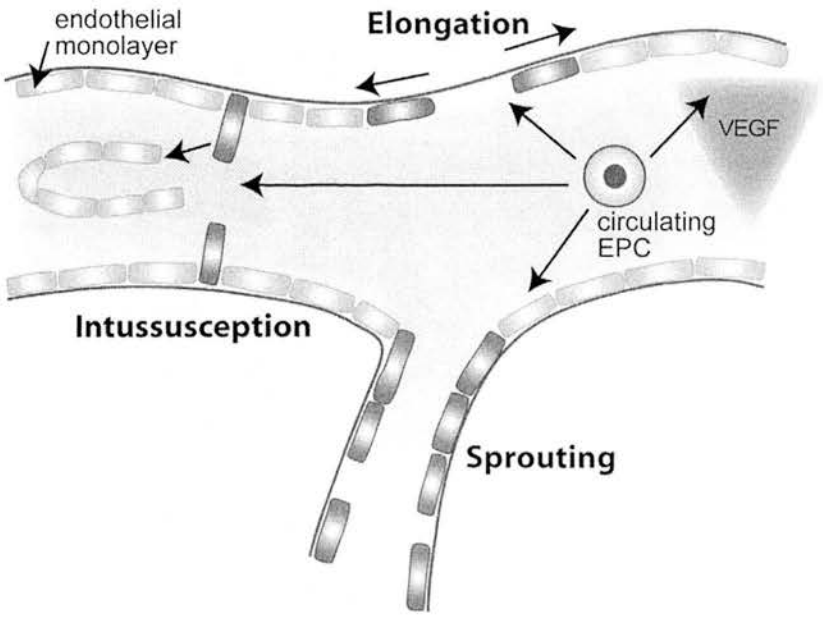

Figure 3 Proposed mechanisms by which EPCs may contribute to angiogenesis in human endometrium. Mature resident endothelial cells (purple) and proliferating mature resident mature endothelial cells (blue). Circulating EPCs (yellow) may incorporate into endothelial monolayer at points of elongation and intussusception, or proliferate to form new micro vessels (sprouting). Circulating EPCs may have a supportive paracrine effect on adjacent mature endothelial cells, through the release of angiogenic factors including VEGF. EPCs release VEGF *in vitro* and in *in vivo* murine studies (Urbich *et al.* 2005). Adapted with permission from Gargett CE & Rogers PA 2001 Human endometrial angiogenesis. *Reproduction* **121** 181–186.

vascular resistance falls resulting in an increase in cardiac output (Ginsburg & Duncan 1967, Ueland *et al.* 1969). The plasma volume expands (Pirani *et al.* 1973). In normal pregnancy, there is enhanced endothelial function and reactivity with greater production of endothelium–derived vasodilators (Goodman *et al.* 1982, Williams *et al.* 1997, Anumba *et al.* 1999). Flow-mediated dilation of the brachial artery increases with the gestational week suggesting maintenance of endothelial vascular function (Dorup *et al.* 1999).

Endothelial function is preserved in spite of the systemic inflammatory response associated with normal pregnancy. There is increased production of pro-inflammatory cytokines, including interleukin (IL)-6, IL-12, and tumour necrosis factor (TNF)-α with associated leucocytosis. The neutrophil count rises steadily throughout gestation, to peak at term (Austgulen *et al.* 1994, Rebelo *et al.* 1995, Melczer *et al.* 2003, Sacks *et al.* 2003). As well as being a pro-inflammatory state, normal pregnancy is associated with increased insulin resistance and hyperlipidaemia, controlled by hormonal changes. Pregnancy might be considered a 'stress' test for the maternal vascular endothelium (Sattar & Greer 2002).

Potential role of EPCs in normal pregnancy

Only three cross-sectional studies of circulating EPCs during normal human pregnancy have been reported to date. Different methods have been used to characterise and quantify putative endothelial progenitors, preventing straightforward comparisons between studies. The first, by Sugawara *et al.* (2005*a*) studied circulating EPCs in the peripheral blood of 20 pregnant women. They observed greater numbers of colony-forming units (EPC-CFUs) at greater gestational age. EPC-CFUs correlated with serum oestradiol concentrations (Sugawara *et al.* 2005*a*). Oestrogens are known to have vasculoprotective effects, in part due to increasing production of nitric oxide and by decreasing reactive oxygen species (Mendelsohn & Karas 1999). Oestrogens also mobilise EPCs from the bone marrow *in vivo*. They inhibit the senescence of EPCs and stimulate VEGF production *in vitro* (Strehlow *et al.* 2003, Imanishi *et al.* 2005). Mobilisation of EPCs may be an important mechanism by which oestrogens protect the vascular endothelium during pregnancy.

A second study by Gussin *et al.* (2002) supports this hypothesis. They cultured peripheral blood mononuclear cells from non-pregnant and pregnant women. Early outgrowth endothelial cells were formed from both groups. Late outgrowth cells, which have a higher proliferative potential, were only formed by the cells from pregnant women. The authors initially hypothesized that these cells were of fetal origin and their original intention was to optimise the culture of fetal cells. To identify fetal cells, they stained for X and Y chromosomes and discovered that none of the colonies contained fetal cells. They concluded that endothelial cells were of maternal origin and that pregnancy is associated with mobilization of EPCs into the circulation (Gussin *et al.* 2002).

In contrast, a study by Matsubara and colleagues of 36 healthy pregnant women observed decreasing numbers of circulating EPCs with increasing gestational age. They directly quantified EPCs by flow-cytometry, selecting for co-expression of CD34, CD133 and KDR. They also assessed EPC proliferation by counting cells that stained for both lectin *Ulex europaeus* and the uptake of

acetylated-LDL after 7 days in culture. With increasing gestation, they found decreased numbers of these cells and decreased responses when stimulated by TNF-α and angiotensin II (Matsubara *et al.* 2006).

It is difficult to compare the results of these studies because different methods were used to count EPCs. It remains uncertain whether the cells measured by flow-cytometry are responsible for forming endothelial-like structures in cell culture. It is possible that the reduction in $CD34^+/CD133^+/KDR^+$ cells is caused by dilution in expanding plasma volume. A limit of all three studies is the cross-sectional study design. Many factors affect circulating EPC numbers were not described for the study subjects. Prospective studies following women through gestation would provide more information about EPCs in pregnancy.

Vascular dysfunction and EPCs in diseases of pregnancy

Pre-eclampsia and gestational diabetes are associated with maternal endothelial dysfunction (McCarthy *et al.* 1993, Knock *et al.* 1997). Pre-eclampsia is the only disease of pregnancy in which EPCs have been studied. Pre-eclampsia, occurring in 5–7% of first pregnancies with 20–25% recurrence is the most common medical complication of pregnancy. The disease, characterised by hypertension and proteinuria, is a significant cause of maternal and perinatal morbidity and mortality worldwide (Stone *et al.* 1994).

Despite recent advances, the pathogenesis of pre-eclampsia remains unclear. Deficient placentation (decreased trophoblast invasion and subsequent spiral artery remodelling) is seen histologically in most women with pre-eclampsia (Brosens *et al.* 1972, Pijnenborg *et al.* 1991). Maternal endothelial dysfunction is a core feature of pre-eclampsia. Vascular cellular adhesion molecule-1 (VCAM), intercellular adhesion molecule-1 (ICAM), E-selectin, endothelin-1 and cellular fibronectin, all soluble markers of endothelial dysfunction are raised in the blood of women with pre-eclampsia. Some are evident before the clinical features of the disease (Taylor *et al.* 1991, Schiff *et al.* 1992, Kraayenbrink *et al.* 1993, Higgins *et al.* 1998, Bretelle *et al.* 2001). Other markers of endothelial dysfunction including asymmetric dimethylarginine (an endogenous inhibitor of nitric oxide synthesis) (Savvidou *et al.* 2003), plasminogen activator inhibitor type 1 (PAI-1) (Roes *et al.* 2002) and tissue plasminogen activator (t-PA) (Belo *et al.* 2002), also rise before clinical symptoms appear with t-PA correlating with the degree of proteinuria (Belo *et al.* 2002). Women with pre-eclampsia are more likely to have impaired uterine artery doppler waveforms (Campbell *et al.* 1983) and reduced flow-mediated dilation of the brachial artery at 23–25 weeks gestation, suggesting that endothelial dysfunction precedes pre-eclampsia (Savvidou *et al.* 2003). Endothelial dysfunction, observed in pre-eclampsia, persists beyond pregnancy (Lampinen *et al.* 2006) and epidemiological data suggest an increased maternal risk of hypertension, coronary and cerebro-vascular disease (Wilson *et al.* 2003).

Pre-existing conditions associated with endothelial dysfunction, such as hypertension, renal disease, and diabetes, increase the risk of developing pre-eclampsia. Although there are extensive studies reporting decreased levels of EPCs or abnormal function of EPCs in men and non-pregnant women with these conditions, there are few available data about EPCs in pre-eclampsia. In addition, as with the studies in normal pregnancy, the published data are conflicting. Matsubara and colleagues reported no difference in the number of circulating EPCs measured by flow-cytometry in pre-eclamptic women although culture of mononuclear cells resulted in more endothelial-like cells. These cells had increased proliferative response following stimulation with TNF-α and angiotensin II compared with cells from women without pre-eclampsia (Matsubara *et al.* 2006). This increase may be a physiological response to ischaemia in the placenta and other organs, similar to that seen in myocardial infarction (Leone *et al.* 2005, Massa *et al.* 2005). In contrast, Sugawara *et al.* (2005*b*) demonstrated decreased numbers of EPC-CFUs and increased senescence of EPCs in patients with pre-eclampsia compared with gestationally matched controls (Sugawara *et al.* 2005*b*). These studies are not easily comparable because of the different methods used, and it is difficult to draw a conclusion from the discordant observations. It is likely that EPC function is more important than quantity, and ideally a subsequent study of EPCs in pre-eclampsia would assess number and function prospectively prior to the onset of disease.

Postulated role of EPCs in pre-eclampsia

The role of EPCs in the pathogenesis of pre-eclampsia is as yet unknown. However from studies outside of pregnancy, EPCs have a potential role in maintaining vascular integrity. Decrease in number and function is associated with endothelial impairment. We hypothesise that EPCs represent a common cellular pathway linking cardiovascular risk factors and endothelial dysfunctional states, with pre-eclampsia. They may be involved in homeostasis of the pregnant uterine and systemic vasculature. Their deficiency or impairment may render the individual vulnerable to the inflammatory and metabolic insults of pregnancy, leading to widespread endothelial dysfunction and pre-eclampsia. Fig. 4 represents this hypothesis schematically.

Future directions

Future research strategies evaluating the role of EPCs in pregnancy will hopefully address the following

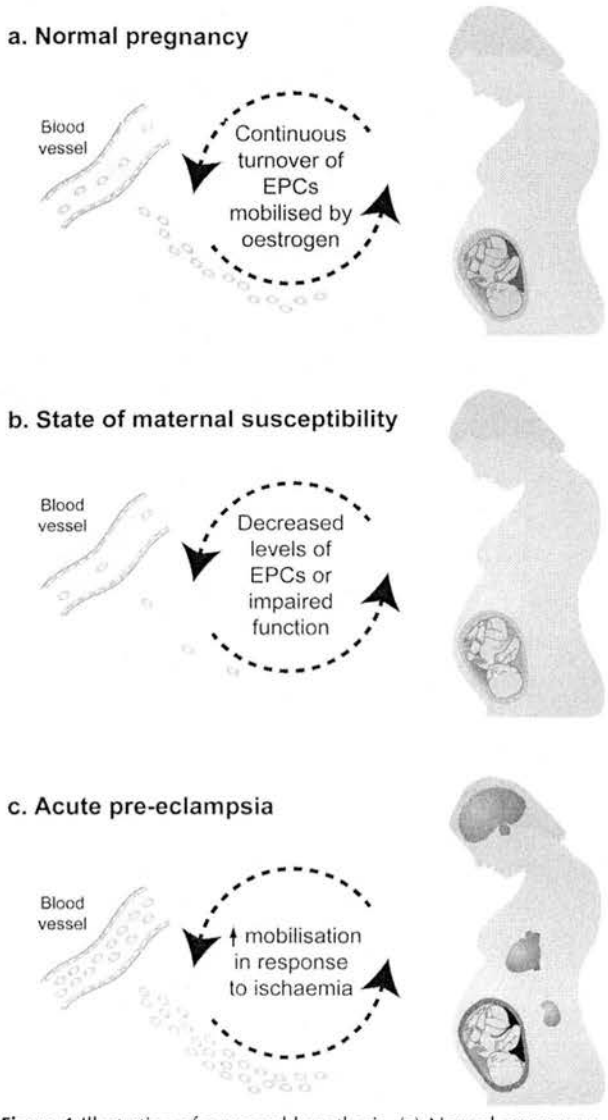

Figure 4 Illustration of proposed hypothesis. (a) Normal pregnancy – there is a continuous demand for endothelial regeneration with vascular adaptation, which occurs in the presence of inflammation. Oestrogens mobilise EPCs from the bone marrow. (b) State of maternal susceptibility – reduced mobilisation of EPCs from the bone marrow or their functional impairment results in maternal susceptibility to endothelial dysfunction. (c) Acute pre-eclampsia – EPCs are mobilised from the bone marrow in response to ischaemia.

questions: Are EPCs useful markers or predictors for pre-eclampsia? Do levels reflect severity of disease?

EPCs may also have a role in other pathologies of pregnancy such as gestational diabetes. Prospective longitudinal studies are required to assess EPC quantity and function during and after pregnancy. In addition, animal studies and *in vitro* models will provide us with a greater understanding of the role of EPCs in the maintenance of the endometrial vasculature and in placentation.

Summary

The discovery of a circulating endothelial progenitor cell that is capable of contributing to physiological and pathological angiogenesis has generated intense interest in the field of vascular biology. To date this has focused particularly in the areas of vascular injury and atherosclerosis where the therapeutic possibilities of EPCs are already being explored. We have summarised the current literature on EPCs in pregnancy and postulate that these progenitors may have an important role in regulating vascular homeostasis in pregnancy. Their impairment or deficiency may underlie maternal susceptibility to pre-eclampsia.

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Methods

Subjects

Ten healthy primigravida women in the third trimester of pregnancy were recruited from community antenatal clinics. Twenty healthy non-pregnant women with at least a 2-month history of normal regular menstrual cycles were recruited from the University of Edinburgh staff and student populations. Exclusion criteria included current or past hypertension, diabetes mellitus, and the use of regular medication other than antacids, inhaled medication for asthma or vitamin supplementation. Non-pregnant women were also excluded if they used hormonal contraception, or had a history of menorrhagia or dysmennorhoea.

All subjects gave written informed consent and the study was approved by the Lothian Research Ethics Committee and undertaken in accordance with the Declaration of Helsinki.

Study protocol

Non-pregnant women attended for a single visit in the mid-follicular phase (day 9.8 ± 0.3) of their menstrual cycle. Pregnant women attended for a single visit during the third trimester of pregnancy (week 36 ± 1).

Studies were carried out in a quiet temperature controlled room (22–25 °C). Subjects fasted for 4 h prior to the study and avoided cigarettes, caffeine and alcohol for the preceding 24 h. The study was conducted in the supine position for non-pregnant women but pregnant women had a slight left lateral tilt to minimize caval compression by the gravid uterus. Blood pressure and heart rate were recorded throughout the study using a semi automated non invasive oscillometric sphygmomanometer (OMRON 705 IT, Kyoto, Japan).

Venous occlusion plethysmography

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [7].

All subjects underwent unilateral brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. Following a 20-min saline infusion, bradykinin at 100, 300 and 1000 pmol min^{-1} (endothelium-dependent vasodilator that stimulates the release of t-PA) and sodium nitroprusside at 2, 4 and 8 μg min^{-1} (endothelium-independent vasodilator that does not stimulate release of t-PA) were infused [7]. The two vasodilators were separated by a 20-min saline infusion and given in a randomized order.

Assessment of acute t-PA release

Venous cannulae (17 gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [7]. Blood (10 mL) was drawn simultaneously from both arms at baseline and after each dose of bradykinin into acidified buffered citrate (Stabilyte tubes, Biopool International, Bray, Ireland; for t-PA assays) and into citrate (BD Vacutainer; for PAI-1 assays). Samples were kept on ice before being centrifuged at 2000 × *g* for 30 min at 4 °C. Platelet-free plasma was decanted and stored at −80 °C before assay. Plasma t PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville Sur Oise, France) concentrations were determined by enzyme-linked immunosorbent assays.

Serum hormonal assays

Venous blood (10 mL) collected in a serum gel tube (Sarstedt Monovette, Germany), was centrifuged and serum samples were stored at −80 C until analysis. Serum concentrations of estradiol, progesterone, lutenizing hormone (LH) and follicle stimulating hormone (FSH) were measured using immunoassay systems (Siemens Medical Solutions Centaur, Camberley, UK, and Abbott Architect, North Chicago, IL, USA).

Data and statistical analysis

Plethysmographic data and net t-PA release were determined as described previously [4,7]. In brief, data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 11.3; Microsoft Corp., Redmond, WA, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes. The last five linear flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the hematocrit, Hct and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\ PA]_{Inf}$) and non-infused ($[t\text{-}PA]_{Noninf}$) arms:

$$\text{Estimated net t-PA release} = \text{FBF} \times (1 - \text{Hct}) \times ([\text{t-PA}]_{\text{Inf}} - [\text{t-PA}]_{\text{Noninf}})$$

The t-PA/PAI-1 ratio was calculated from the respective antigen concentrations. Continuous variables are reported as mean ± SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, La Jolla, CA, USA) using analysis of variance (ANOVA) with repeated measures and two tailed Student's *t*-test where appropriate. Statistical significance was taken at two-sided $P < 0.05$.

Results

Baseline characteristics

Due to technical problems, one pregnant woman and three non pregnant women were unable to complete the complex

vascular studies. Pregnant (n = 9) and non-pregnant (n = 17) women were well matched for age (26 ± 3 vs. 27 ± 1, P = 0.67) and body mass index (24 ± 1 vs. 24 ± 1 kg m^{-2}, P = 0.77). Serum hormonal assays confirmed that the non-pregnant women were studied in the mid-follicular phase of their menstrual cycle (data not shown). All pregnant women had uncomplicated pregnancies and delivered at term (mean gestational age at delivery of 40 ± 1 weeks) with average birth weights (3144 ± 186 g). Three pregnant and three non-pregnant women were unable to complete the fibrinolytic studies due to technical problems with simultaneous venous access, leaving a final study group of six pregnant and 14 non-pregnant women for fibrinolytic analysis. These women were well matched for age (26 ± 4 vs. 27 ± 2, P = 0.94), body mass index (24 ± 1 vs. 24 ± 1 kg m^{-2}, P = 0.85) and current smoking status (2/6 vs. 3/14, P = 0.6).

Vasomotor function There were no differences in resting systolic (122 ± 3 vs. ± 2, P = 0.14) or diastolic blood pressure (76 ± 3 vs. 73 ± 2, P = 0.39) between pregnant and non-pregnant women. Similarly, there was no difference in basal infused (3.1 ± 0.5 vs. 2.1 ± 0.2, P = 0.07) or non-infused (2.4 ± 0.4 vs. 1.8 ± 0.2, P = 0.11) forearm blood flow between pregnant and non-pregnant women. However, consistent with a hyperdynamic circulation, pregnant women had increased resting heart rates compared with non-pregnant women (80 ± 3 vs. 67 ± 2 beats min^{-1}, P = 0.002).

There was a dose-dependent increase in forearm blood flow with bradykinin (P < 0.0001 for both) and sodium nitroprusside ($P \leq 0.002$ for both). Endothelium-dependent and endothelium-independent vasodilatation was similar between the two groups of women (P = 0.6 and P = 0.8 respectively, two-way ANOVA with repeated measures; Table 1).

Fibrinolytic function Baseline plasma PAI-1 antigen concentrations were higher in pregnant women (77.1 ± 12.4 vs. 21.5 ± 9.8 ng mL^{-1} in non-pregnant women; P = 0.004, unpaired t-test; Table 2) whilst apparently higher plasma t-PA antigen concentrations did not achieve statistical significance (13.5 ± 2.4 vs. 7.5 ± 2.0 ng mL^{-1}, respectively; P = 0.10, unpaired t-test). Consistent with this, t-PA/PAI-1 ratios (0.2 ± 0.1 vs. 0.6 ± 0.1; P = 0.02) and plasma t-PA activity concentrations (0.17 ± 0.02 vs. 0.58 ± 0.06 IU mL^{-1}; P < 0.0004, unpaired t-test) were lower in pregnant women.

Bradykinin caused a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused arm of both pregnant and non-pregnant women ($P \leq 0.005$ for all; Table 2). The increase in t-PA activity was greater in the non-pregnant women (P = 0.02 vs. pregnant; two-way ANOVA; Fig. 1). Bradykinin increased the net release of t-PA antigen and activity in both pregnant women and non-pregnant women (P < 0.05 for all; Table 2). Both net release of active t-PA and plasma t-PA/PAI-1 ratios were markedly reduced in pregnant women (P < 0.05 for both; Table 2 and Fig. 2). Compared with the non-pregnant women, the area under the curve for net active t-PA release was reduced by 36% in the pregnant women.

Discussion

In the third trimester of pregnancy, we have demonstrated that there is perturbation of the fibrinolytic system. Consistent with previous work [8,9], we report a marked increase in basal plasma PAI-1 concentrations leading to a reduction in t-PA activity and t-PA/PAI-1 ratios. For the first time, we have assessed acute endothelial t PA release in pregnant women. Despite normal bradykinin-induced vasodilatation and release of t-PA antigen, there was a major inhibition of the acute increase in t-PA activity that was primarily attributable to an excess of PAI-1. We conclude that this inadequate acute fibrinolytic activity contributes to the prothrombotic consequences of pregnancy.

Pregnancy is associated with a marked elevation in plasma PAI-1 concentrations that, in our study, were 4–5-fold higher than age matched non pregnant women and comparable with concentrations found in previous studies [1]. Although there appeared to be a modest concomitant rise in t PA antigen, this

Table 1 Absolute forearm blood flow responses to endothelium-dependent and independent vasodilators

	Non-pregnant				Pregnant			
Bradykinin pmol min^{-1}	0	100	300	1000	0	100	300	1000
FBF, mL 100 mL^{-1} min^{-1}								
Non-infused arm	2.0 ± 0.2	2.0 ± 0.3	1.8 ± 0.3	2.0 ± 0.3	3.1 ± 0.6	3.2 ± 0.6	3.6 ± 0.7	3.3 ± 0.6
Infused arm	2.8 ± 0.4	10.1 ± 1.0	13.2 ± 1.2	19.1 ± 1.9*	3.9 ± 0.6	11.2 ± 1.7	15.3 ± 2.5	18.7 ± 2.4*
	Non-pregnant				Pregnant			
Sodium nitroprusside μg min^{-1}	0	2	4	8	0	2	4	8
FBF, mL 100 mL^{-1} min^{-1}								
Non-infused arm	2.4 ± 0.4	2.4 ± 0.5	2.3 ± 0.4	2.1 ± 0.4	2.9 ± 1.1	3.1 ± 1.2	2.7 ± 1.0	4.3 ± 1.2
Infused arm	3.7 ± 0.9	10.6 ± 1.2	13.1 ± 1.4	16.0 ± 1.9*	4.0 ± 1.4	10.6 ± 2.5	11.2 ± 2.6	14.8 ± 3.2*

Data expressed as mean ± SEM for nine pregnant and 17 non-pregnant women. *P < 0.0001 for bradykinin, *P = 0.002 and P < 0.0001 for sodium nitroprusside (pregnant and non-pregnant groups, respectively) one-way ANOVA.

Table 2 Plasma t-PA and PAI-1 antigen and activity concentrations in both forearms

	Non-pregnant (n = 14)				Pregnant (n = 6)			
Bradykinin pmol min^{-1}	0	100	300	1000	0	100	300	1000
t-PA Antigen, ng mL^{-1}								
Non-infused arm	7.45 ± 2.0	7.6 ± 2.0	8.0 ± 2.3	9.0 ± 2.8	13.5 ± 2.4	13.6 ± 2.6	14.6 ± 3	15.8 ± 3.2
Infused arm	6.95 ± 2.0	8.6 ± 2.0	12.2 ± 2.5	16.0 ± 3.0	13.4 ± 2.7	12.9 ± 2.4	13.7 ± 2.3	19.2 ± 2.0
t-PA Activity, IU mL^{-1}								
Non-infused arm	0.58 ± 0.06	0.6 ± 0.08	0.79 ± 0.09	1.1 ± 0.14	0.17 ± 0.02	0.26 ± 0.06	0.43 ± 0.2	0.53 ± 0.12
Infused arm	0.5 ± 0.06	1.6 ± 0.19	2.8 ± 0.35	4.3 ± 0.6	0.51 ± 0.32	0.51 ± 0.1	1.3 ± 0.15	2.4 ± 0.31
PAI-1 Antigen, ng ml^{-1} mL^{-1}								
Non-infused arm	21.5 ± 9.8	23.2 ± 10.3	22.9 ± 9.6	26.9 ± 10.0	77.1 ± 12.4	71.8 ± 6.3	80.5 ± 11.4	78.5 ± 5.8
Infused arm	19.2 ± 7.7	18 ± 7.3	18.8 ± 7.32	19.8 ± 7.4	72.6 ± 9.0	87.1 ± 16.3	82.8 ± 14.0	82.9 ± 3.1
PAI-1 Activity, ng ml^{-1} mL^{-1}								
Non-infused arm	2.1 ± 1.8	1.4 ± 1.0	1.1 ± 0.9	1.1 ± 0.8	5.7 ± 1.4	6.1 ± 1.7	6.2 ± 1.7	5.8 ± 1.6
Infused arm	1.36 ± 1.0	1.31 ± 1.0	1.9 ± 1.7	1.1 ± 0.9	5.7 ± 1.5	5.9 ± 1.7	5.8 ± 1.7	6.6 ± 2.5
Net t-PA Antigen release ng 100 mL^{-1} of tissue mm^{-1}	−0.05 ± 0.8	12.2 ± 3.19	62.3 ± 13	150.3 ± 32.6	−2.8 ± 3.03	0.07 ± 8.0	6.63 ± 15.8	101.6 ± 59
Net tPA activity release IU 100 mL^{-1} of tissue mm^{-1}	0.17 ± 0.07	8.8 ± 1.3	24.7 ± 2.7	60.8 ± 11.2	0.85 ± 1.0	3.4 ± 2.0	17.2 ± 7.4	41.3 ± 13.1

Data expressed as mean ± SEM for six pregnant and 14 non-pregnant subjects. Fibrinolytic data not available or incomplete for three pregnant and three non-pregnant subjects due to technical difficulties with simultaneous cannulation during study.

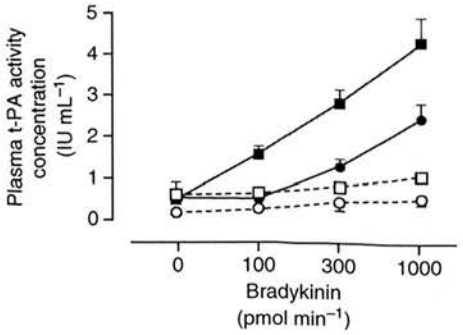

Fig. 1. Plasma tissue plasminogen activator (t-PA) activity concentrations in the infused (solid symbols and lines) and non-infused (open symbols and dashed lines) arms of pregnant (circles) and non-pregnant (squares) women during intra-arterial bradykinin infusion. Plasma t-PA activity increased in the infused arm of both pregnant and non-pregnant women (P < 0.001 for both; one-way ANOVA) but was greater in non-pregnant women (P = 0.02 vs. pregnant women; two-way ANOVA).

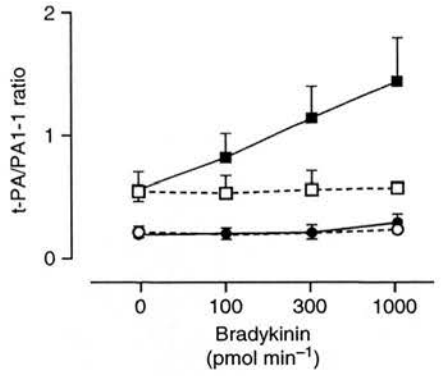

Fig. 2. Ratio of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) in the infused (solid symbols and lines) and non infused (open symbols and dashed lines) arms of pregnant (circles) and non pregnant (squares) women during intra-arterial bradykinin infusion. Plasma t-PA/PAI-1 ratios were markedly lower in pregnant women (P = 0.04, vs. non-pregnant women; two-way ANOVA).

was unable to compensate for the dominance of PAI-1 as demonstrated by the adverse effect on the ratio of t-PA to PAI-1. Thus, while we were able to stimulate the release of t-PA from the endothelium using intra arterial bradykinin, the overwhelming effect of PAI-1 markedly inhibited the plasma activity of both basal and stimulated t-PA. This marked shift in fibrinolytic potential, and the associated dramatic reduction in active t-PA, will have prothrombotic consequences for pregnant women.

Pregnancy is associated with an increased risk of systemic thrombotic events including thromboembolism and myocardial infarction. Although rare (six per 100 000 pregnancies), the age-adjusted incidence of acute myocardial infarction is increased 4-fold by pregnancy. Acute myocardial infarction in women has several features that distinguish it from those seen in men. In a half of women, myocardial infarction results from plaque erosion rather than rupture and, in some instances, endothelial erosion and coronary thrombosis can occur in the absence of atherosclerosis [10]. Thus, thrombosis and prothrombotic states are more likely to be implicated in the pathogenesis of acute myocardial infarction in women and this may be mediated through changes in fibrinolytic function.

In the current study we have demonstrated a reduction of bradykinin-induced release of active t-PA and t-PA/PAI-1 ratio in normal pregnancy compared with non-pregnant women. This is consistent with a study by Segar *et al.* [11] that demonstrated reduced t-PA activity in pregnant compared with non-pregnant women after the venous occlusion test.

Moreover these findings mirror that of a previous study in which we demonstrated that current smokers had elevated basal plasma t-PA and PAI-1 antigen concentrations that were associated with reduced basal and stimulated active t-PA release [12]. Coronary thrombosis is one of the major causes of sudden cardiac death in both smokers [13] and myocardial infarction during pregnancy [14]. The alteration in fibrinolytic function that we have demonstrated may therefore provide a common pathogenic mechanism underlying coronary thrombosis in both patient populations. Moreover, it may in part explain the strong association of myocardial infarction with hypertension during pregnancy [5].

In conclusion, pregnancy is associated with perturbations of endogenous fibrinolytic capacity that is due an overwhelming increase in plasma PAI-1 concentrations, in the presence of unchanged t-PA antigen release leading to inadequate active t-PA. These pro-thrombotic effects may, in part, explain the increased risk of arterial and venous thrombosis both systemically and locally within the feto-placental unit in pregnant women.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Influence of the Menstrual Cycle, Pregnancy, and Preeclampsia on Arterial Stiffness

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Abstract — Arterial stiffness and compliance are major predictors of adverse cardiovascular events and are influenced by female sex hormones, including estrogen and progesterone. The aim of this longitudinal study was to evaluate the effect of the menstrual cycle, normal pregnancy, and preeclampsia on central and systemic arterial stiffness. Ten healthy nulliparous women with regular menses were studied in the early and midfollicular, periovulatory, and luteal phases of a single menstrual cycle. Twenty-two primigravida pregnant women were studied throughout pregnancy at 16, 24, 32, and 37 weeks gestation and at 7 weeks postpartum. Fifteen primigravida women with preeclampsia were studied at diagnosis and 7 weeks postpartum. Augmentation index and carotid radial and carotid-femoral pulse wave velocities were measured using applanation tonometry. Augmentation index fell during the luteal phase of the menstrual cycle (luteal phase versus periovulatory phase; $P<0.05$). In normal pregnancy, pulse wave velocity and augmentation index increased from 24 weeks over the third trimester ($P\leq0.01$ for both). All of the measures were increased in women with preeclampsia ($P\leq0.01$), with augmentation index and carotid femoral pulse wave velocity remaining elevated 7 weeks postpartum ($P\leq0.02$). We conclude that systemic arterial stiffness undergoes major changes during the menstrual cycle and pregnancy and that preeclampsia is associated with greater and more prolonged increases in arterial stiffness. These effects may contribute to adverse cardiovascular outcomes of pregnancy and preeclampsia. **(*Hypertension*. 2009;53: 00-00.)**

Key Words: hypertension ■ arterial stiffness ■ pregnancy ■ preeclampsia ■ menstrual cycle

Arterial stiffness is a key determinant of central aortic pressure and is an independent predictor of adverse cardiovascular outcomes and organ damage.[1,2] Female sex affects arterial stiffness that is mediated in part via the influence of both estrogen and progesterone on arterial structure and function.[3] In the prepubertal and postmenopausal years, when female sex steroids are low, women have stiffer arteries than age matched men.[4,5] During the reproductive years, female sex steroids fluctuate cyclically during the menstrual cycle and increase dramatically in pregnancy. The initial effect of pregnancy reducing arterial stiffness is well documented in both animal and human studies.[6–11] However, data are conflicting concerning the effect of sex steroids on arterial stiffness during the menstrual cycle[12,13] and the effect of later gestation.[14,15]

Augmentation index and pulse wave velocity (PWV) are the principal measures of central arterial pressure and stiffness that can be determined noninvasively using applanation tonometry. Within normotensive pregnancy, PWV is more closely associated with birth weight than mean arterial pressure, suggesting that arterial stiffness may represent maternal adaptation to pregnancy better than blood pressure.[16] Preeclampsia is a common hypertensive complication of pregnancy, which causes significant maternal and fetal morbidity. Inadequate cardiovascular adaptation in early pregnancy may predate its clinical presentation,[17] and it is associated with an increased long-term risk of maternal cardiovascular disease. Understanding the relationship between preeclampsia and arterial stiffness may, therefore, not only inform understanding of its pathogenesis but may also increase our understanding of the association between preeclampsia and later cardiovascular disease. The aim of the current longitudinal study was, therefore, to evaluate the effects of the menstrual cycle, normal pregnancy, and preeclampsia on central and systemic arterial stiffness.

Methods

Subjects

Healthy premenopausal nonsmoking nulliparous women (n=10) with at least a 2-month history of normal regular menstrual cycles were recruited to the study. All of the study group had confirmed ovulation, defined as day 21 serum progesterone >30 nmol/L (please see the online data supplement at http://hyper.ahajournals.org). Exclusion criteria included current or past hypertension, use of hormonal contraception, or use of regular medication. Nonsmoking healthy

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primigravida women with an uncomplicated singleton pregnancy (n=22) were recruited in the first trimester of pregnancy. Exclusion criteria included current or past hypertension, the use of regular medication, and the development of complications during pregnancy. Women with a singleton pregnancy who fulfilled the diagnostic criteria for preeclampsia, as defined by the International Society for the Study of Hypertension in Pregnancy, were recruited at diagnosis (n=15).[18] Exclusion criteria included pre-existing hypertension.

All of the subjects gave written informed consent, and the study was approved by the Lothian Research Ethics Committee and undertaken in accordance with the Declaration of Helsinki. All of the procedures followed were in accordance with institutional guidelines.

Visit Schedule

Nonpregnant women attended for 4 visits during a single menstrual cycle: early follicular (days 1 to 3), midfollicular (days 6 to 8), periovulatory (days 13 to 15), and luteal (days 20 to 22) phases. Women with uncomplicated pregnancies attended for 4 visits during pregnancy (16, 24, 32, and 37 weeks) and 1 visit at 7 weeks postpartum. Women with preeclampsia attended after diagnosis and at 7 weeks postpartum.

Study Protocol

At each visit, all of the subjects abstained from alcohol and caffeine for 12 hours and fasted for 4 hours before attendance. All of the subjects had an initial rest period of 30 minutes in a quiet, temperature-controlled room. Nonpregnant subjects rested in the supine position, whereas pregnant subjects rested in the 30° left lateral position to avoid inferior vena cava compression by the gravid uterus. In both groups, all of the subsequent measurements were done in the supine position. Heart rate, blood pressure (recorded in duplicate using an automated sphygmomanometer; Microlife 3BTO-A, validated for use in pregnancy and preeclampsia),[19] and augmentation index were measured on all of the subjects at every visit. PWV was performed at the early follicular visit for nonpregnant subjects and at every visit in women with an uncomplicated pregnancy or with preeclampsia.

Augmentation Index

Applanation tonometry of the radial artery was performed using a micromanometer (Millar Instruments) and the SphygmoCor system (AtCor Medical) in accordance with the manufacturer's recommendations. An aortic pulse pressure waveform was derived from the radial artery waveform via a mathematical transfer function. From this, the augmentation index (defined as the difference between the second and first systolic peaks, expressed as a percentage of the pulse pressure) was calculated (please see the online data supplement). The SphygmoCor system also reports augmentation index corrected for a heart rate of 75 bpm. The augmentation index is a measure of systemic arterial stiffness and wave reflection. Arterial blood pressure varies with respiration; thus, to cover a complete respiratory cycle, ≥2 independent analyses, incorporating 10 arterial waveforms each, were obtained and averaged from each subject.

Pulse Wave Velocity

Using the same equipment, carotid-femoral PWV and carotid-radial PWV were determined by sequential acquisition of pressure waveforms from the carotid, femoral, and radial arteries. The timing of these waveforms was compared with the R wave on the simultaneously recorded ECG to calculate the time delay. For each subject, a total of 2 consecutive waveform recordings was obtained, and the mean of 2 PWV readings was recorded.

Staff specifically trained in the technique performed all of the vascular measurements. Our interobserver and intraobserver variabilities have been described previously.[20] Only measurements meeting SphygmoCor quality control criteria were accepted.

Table 1. Baseline Characteristics of Study Population

Baseline Characteristics	Nonpregnant Group (n=10)	Healthy Pregnant Group (n=22)	Preeclamptic Group (n=15)	
			Preterm (n=7)	Term (n=8)
Age, y	31±2	30±1	30±4	30±2
Height, m	1.70±0.02	1.65±0.02*	1.63±0.02*	1.62±0.02*
Weight, kg	68±3	73±3	66±4	75±4
Body mass index, kg/m^2	23±1	27±1	25±2	29±1†
Gestation at delivery, d	NA	285±2	213±10‡	270±3‡
Gestation at delivery, wk	NA	41±0.3	30±1.4‡	39±0.4‡
Birth weight, g	NA	3497±112	1371±222§	3231±252

Body mass index was recorded during first trimester for pregnant subjects. Data are reported as mean±SEM. NA indicates not applicable.

*$P<0.05$ vs nonpregnant group.

†$P\leq0.0009$ vs nonpregnant group.

‡$P\leq0.0003$ vs healthy pregnant group.

§$P<0.0001$ vs healthy pregnant and term groups.

Measurement of Soluble Hormones

At each visit, peripheral venous blood was drawn from a large antecubital vein. Serum was prepared from blood collected into serum gel tubes (Sarstedt Monovette) and stored at −80°C until analysis. Estradiol and progesterone were measured using the Siemens Medical Solutions Centaur immunoassay system, and luteinizing hormone and follicle-stimulating hormone concentrations were measured in samples from the nonpregnant women using the Abbott Architect immunoassay system.

Statistical Analysis

Continuous variables were analyzed using the Kolmogorov-Smirnov test for normality and reported as mean±SEM. For comparisons across the menstrual cycle and within healthy pregnancy, analyses were performed using 1 way ANOVA with repeated measures and Bonferroni's post-tests. Two-tailed paired Student *t* tests were used when comparing pregnant and postpartum data within a subject group. Two-tailed unpaired Student *t* tests were used when comparing data between different subject groups. All of the calculations were performed using GraphPad Prism (GraphPad Software). Statistical significance was taken at 5%.

Results

Baseline Characteristics

Baseline demographics for the nonpregnant (n=10) and pregnant (women with uncomplicated pregnancy: n=22; women with preeclampsia: n=15) study groups, and longitudinal hemodynamic variables are presented in Table 1. Women with preeclampsia were classified according to gestation at presentation, into preterm (n=7; presented before 34 weeks; mean: 30; range: 24 to 34 weeks) and term (n=8; presented after 34 weeks; mean: 38; range: 36 to 40 weeks) groups, respectively.[21] The mean gestations at delivery for women in the preterm and term groups were 30.3 weeks (range: 24.0 to 35.8) and 38.6 weeks (range: 36.4 to 40.4), respectively. Nonpregnant and pregnant groups were well matched for maternal age and body mass index; however, pregnant women were shorter in height than those in the nonpregnant group. There were no differences in systolic and

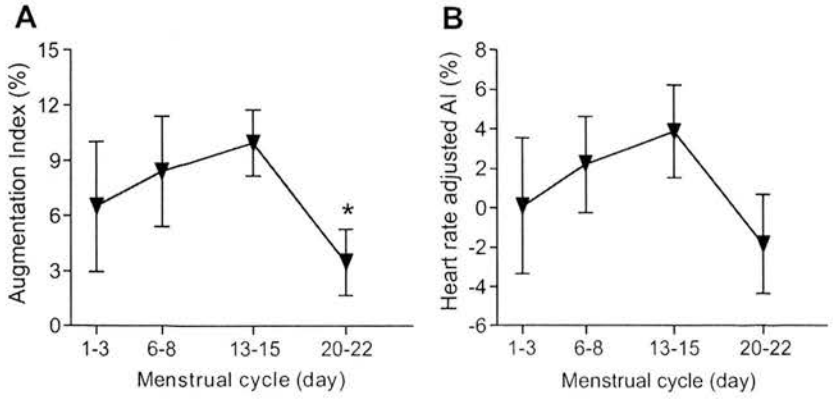

Figure 1. A, Augmentation index throughout menstrual cycle. Augmentation index varied during the menstrual cycle ($P=0.03$) with a fall in the luteal phase compared with the periovulatory phase (*$P<0.05$). Data are reported as mean±SEM. B, Heart rate–adjusted augmentation index throughout menstrual cycle. There was a trend toward a reduction in heart rate adjusted (at 75 bpm) in the luteal compared with the periovulatory phase ($P=0.07$). Data are reported as mean±SEM.

diastolic blood pressures in the first trimester in women who subsequently had an uncomplicated pregnancy or developed preeclampsia (data not shown). At the postpartum visit, although blood pressure had returned to within the normal range in women who had preeclampsia, it was still higher than in those women who had had an uncomplicated pregnancy ($P<0.001$).

In women with preterm preeclampsia, 5 were taking regular labetalol and nifedipine, 1 was taking regular methyldopa and nifedipine, and 1 was receiving no antihypertensive therapy. Six of these women received antenatal betamethasone. In women with term preeclampsia, 1 was taking regular labetalol, with the remaining 7 women not receiving antihypertensive therapy. None received antenatal betamethasone. Postpartum, of the original 15 women who had developed preeclampsia, only 3 women were taking labetalol, and 1 was taking methyldopa.

Effect of Menstrual Cycle on Augmentation Index

Augmentation index varied over the menstrual cycle ($P=0.03$), with a fall in the luteal phase compared with the periovulatory phase (3.5±1.8% versus 9.9±1.8%; $P<0.05$; Figure 1A and 1B). There were no changes in any other recorded hemodynamic variables throughout the menstrual cycle. There was no correlation between augmentation index and serum estradiol or progesterone at any time point in the cycle.

Effect of Normal Pregnancy on Augmentation Index and PWV

Augmentation index was adjusted for heart rate (calculated at a heart rate of 75 bpm) because of variation in heart rate during pregnancy and postpartum ($P<0.0001$). Heart rate–corrected augmentation index varied with gestation in normal pregnancy ($P<0.0001$; Figure 2) rising toward term (16 weeks versus 37 weeks, 24 weeks versus 37 weeks, and 32 weeks versus 37 weeks; all $P<0.01$). Moreover, heart rate–corrected augmentation index was persistently elevated at 7 weeks postpartum compared with 16 weeks gestation (8.7±1.9% versus −3.0±2.5%; $P=0.0002$).

Both carotid-femoral and carotid-radial PWVs varied with gestation in normal pregnancy (both $P=0.01$; Figure 3A and 3B). Carotid-femoral PWV increased from 24 weeks to 7 weeks postpartum (5.0±0.2 m/s versus 5.5±0.2 m/s; $P=0.0008$). Carotid-radial PWV rose from 16 and 24 weeks to term (16 weeks versus 37 weeks and 24 weeks versus 37 weeks, both 6.4±0.2 m/s versus 7.0±0.2 m/s; $P<0.05$), and values at 7 weeks postpartum were not different from those at term (postpartum versus 37 weeks, 6.6±0.2 m/s versus 7.0±0.2 m/s; $P=0.07$). There was no correlation among augmentation index, carotid femoral PWV, or carotid radial PWV and serum estradiol or progesterone concentrations at any time point in pregnancy.

Effect of Preeclampsia on Augmentation Index and Pulse Wave Analysis

All of the hemodynamic variables differed between the women with preeclampsia and women with uncomplicated pregnancies at similar gestation, apart from heart rate in women with term preeclampsia (Table 2). Augmentation index, carotid-femoral PWV, and carotid-radial PWV were raised in women with both preterm and term preeclampsia compared with gestationally matched women with uncomplicated pregnancies ($P\leq0.001$ for both, Figure 2; $P\leq0.01$ for both, Figure 3A, and $P\leq0.006$ for both, Figure 3B, respectively). There were no differences in augmentation index,

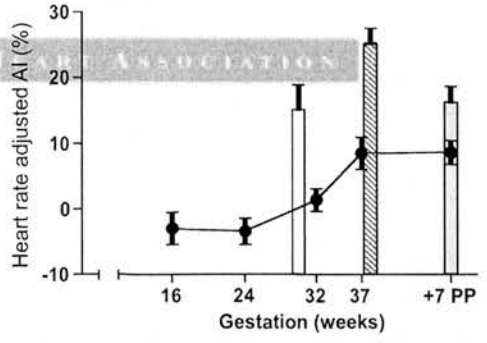

Figure 2. Effect of pregnancy, gestation, and preeclampsia on augmentation index (adjusted for heart rate). Heart rate–adjusted augmentation index varied with gestation in normal pregnancy (circles; $P<0.0001$) rising toward term ($P<0.01$) and was elevated at 7 weeks postpartum compared with 16 weeks gestation ($P=0.0002$). Compared with gestation-matched controls, augmentation index was raised in both preeclamptic groups (preterm and term, white and hatched columns, respectively; both $P\leq0.001$) and remained elevated postpartum (gray column; $P=0.02$). There was no difference in the augmentation index between the 2 preeclamptic groups ($P=0.05$). Data are reported as mean±SEM.

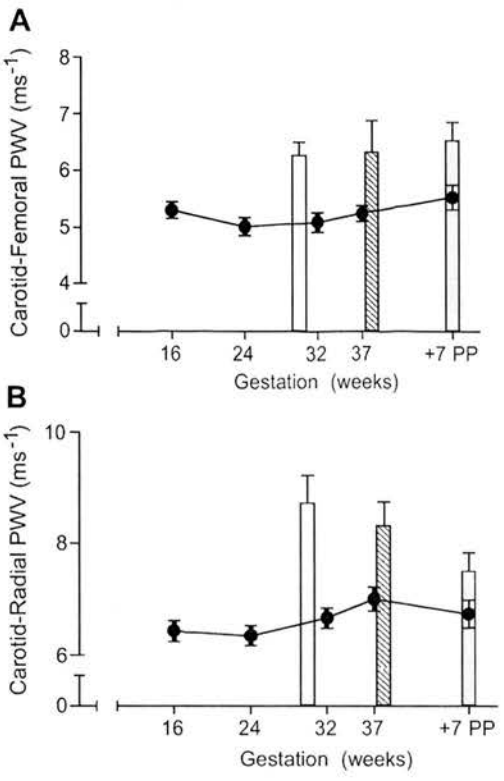

Figure 3. A, Effect of pregnancy, gestation, and preeclampsia on carotid-femoral PWV. Carotid-femoral PWV varied with gestation in normal pregnancy (circles; $P=0.01$). Compared with gestation-matched controls, carotid-femoral PWV was raised in both preeclamptic groups (preterm and term, white and hatched columns, respectively; both $P\leq0.01$) and remained elevated postpartum (gray column; $P=0.01$). There was no difference in carotid-femoral PWV between the 2 preeclamptic groups. Data are reported as mean±SEM. B, Effect of pregnancy, gestation, and preeclampsia on carotid-radial PWV. Carotid-radial PWV varied with gestation during normal pregnancy (circles; $P=0.01$). Compared with gestation-matched controls, carotid-radial PWV was raised in both preeclamptic groups (preterm and term, white and hatched columns, respectively; both $P\leq0.006$). There was no difference in carotid-radial PWV between the 2 preeclamptic groups in pregnancy. Data are reported as mean±SEM.

carotid-femoral PWV, or carotid-radial PWV between women with preterm or term preeclampsia (all $P>0.05$). At the postpartum visit, despite blood pressure returning to within the normal range, augmentation index and carotid-femoral PWV remained elevated at 7 weeks compared with women with uncomplicated pregnancies (16.2±2.5% versus 8.7±1.9% and 6.5±0.3 m/s versus 5.5±0.2 m/s, respectively, $P\leq0.02$ for both; Figures 2 and 3A). In contrast, there was no difference in carotid-radial PWV by 7 weeks postpartum between women with preeclampsia compared with women with uncomplicated pregnancies (7.5±0.3 m/s versus 6.8±0.3 m/s; $P=0.08$; Figure 3B).

Discussion

PWV and augmentation index together provide a comprehensive assessment of arterial function that is highly reproducible and validated in healthy subjects and those with cardiovascular disease.[20] In this longitudinal study, we have demonstrated that augmentation index decreases during the luteal phase of the menstrual cycle before rising at the beginning of the menstrual cycle. During normal pregnancy, arterial stiffness increases from the midtrimester to term. Preeclampsia is associated with increased arterial stiffness and, despite blood pressure returning to within the normal range, this persists in the immediate postpartum period. Increased arterial stiffness, therefore, seems to be a feature of preeclampsia that extends beyond pregnancy and may contribute to the adverse cardiovascular outcomes associated with preeclampsia.

The present study is the first to use the augmentation index to determine the effect of the menstrual cycle on systemic arterial stiffness. We demonstrated that augmentation index is reduced in the luteal phase of the cycle, indicating decreased systemic arterial stiffness. Previous studies have demonstrated either no change[22] or an increase in compliance in the ovulatory phase compared with the follicular and luteal phases.[12,13,23] A variety of factors may account for these seemingly discrepant findings but, in particular, the differing methodologies used, sample population characteristics, and timing of sampling. Our study used the augmentation index as a method of evaluating systemic arterial stiffness, whereas other studies have assessed whole body arterial compliance that combines both central and peripheral measures,[13] or carotid artery compliance, a surrogate for aortic compliance.[12] In our longitudinal study, we demonstrated clear differences in the augmentation index depending on the day of study. This variation will be magnified if a broader sampling window is used, as in the study by Giannattasio et al.[23] Our study group was well characterized, with all of the women ovulating, as indicated by a rise in luteal phase progesterone. Despite this, neither absolute nor change in serum hormone concentrations correlated with change in augmentation index in our study. This perhaps reflects the small numbers of women in our study. Alternatively, it may imply that these hormones do not directly regulate arterial stiffness and that other intermediate factors, eg, the renin-angiotensin[24] or endothelin[25] systems, regulate vascular tone and augmentation pressure during the menstrual cycle.

Because of the logistical difficulties in obtaining prepregnancy data for pregnant women, we compared our pregnancy data with those obtained from the same women 7 weeks postpartum. Although many cardiovascular parameters normalize rapidly over the first 2 weeks postdelivery, many require a longer time frame to settle and probably do not fully recover to preconceptual values.[26] Moreover, Bernstein et al[27] demonstrated that mean arterial pressure is lower in subsequent normal pregnancies than in first pregnancies and that a shorter interpregnancy interval leads to a greater reduction in mean arterial pressure. Together these studies suggest that structural vascular changes occur in pregnancy and persist beyond the gestational period.[27]

Our findings of a rise in arterial stiffness from the second trimester to term and postnatally are supportive of previous studies using brachial-ankle PWV as a composite measure of systemic and central stiffness[28] and augmentation index.[29] Other studies report no change in PWV with gestation[30] or a general decrease in PWV and augmentation index during pregnancy.[6,8,9,11] All of these studies had limited and wide

Table 2. Hemodynamic Variables of Healthy Pregnant Women and Women With Preeclampsia Longitudinally During Pregnancy and Postpartum

	Healthy Pregnant Group (n=22)						Preeclamptic Group (n=15)					
Hemodynamic Variables	16 wk Gestation	24 wk Gestation	32 wk Gestation	37 wk Gestation	Postpartum Visit	Significance, Within Pregnancy (4 Time Points, 16 to 37 wk)	Preterm (30 wk) (n=7)	Term (38 wk) (n=8)	Postpartum Visit	Preterm vs Pregnant Control at 32 wk	Term vs Pregnant Control at 37 wk	Significance, Postpartum Comparisons*
Heart rate, bpm	69±2	72±2	77±2	77±2	59±1	$P<0.0001$	67±3	76±5	65±3	$P=0.02$	$P=0.8$	$P=0.02$
Peripheral SBP, mm Hg	113±2	111±1	113±1	117±1	113±1	$P=0.004$	140±3	146±2	123±3	$P<0.0001$	$P<0.0001$	$P=0.002$
Peripheral DBP, mm Hg	65±1	65±1	70±1	76±1	69±1	$P<0.0001$	87±3	95±2	77±3	$P<0.0001$	$P<0.0001$	$P=0.004$
Peripheral PP, mm Hg	48±2	46±2	43±1	41±1	42±2	$P=0.003$	52±4	52±4	45±3	$P=0.004$	$P=0.001$	$P=0.4$
Central SBP, mm Hg	93±2	92±1	95±1	103±2	98±2	$P<0.0001$	126±4	135±3	111±3	$P<0.0001$	$P<0.0001$	$P=0.0006$
Central DBP, mm Hg	64±1	64±1	69±1	76±1	70±1	$P<0.0001$	89±3	97±2	79±3	$P<0.0001$	$P<0.0001$	$P=0.002$
Central PP, mm Hg	30±1	28±1	26±1	27±1	29±2	$P=0.13$	37±2	38±3	33±2	$P<0.0001$	$P=0.0002$	$P=0.2$
Mean arterial pressure, mm Hg	81±1	80±1	84±1	90±1	84±1	$P<0.0001$	105±2	113±2	92±2	$P<0.0001$	$P<0.0001$	$P=0.001$

PP indicates pulse pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure. Data are reported as mean±SEM.
*Postpartum comparison between all of the women with preeclampsia and healthy pregnant women at 7 weeks postpartum.

time points, with the third-trimester visits performed earlier than in our study. These limitations may potentially explain why the subtle rise in PWV and augmentation index in the third trimester went undetected in these studies.

The relative reduction in arterial stiffness during pregnancy compared with postpartum is likely to arise from several factors. Estrogen has favorable effects on the endothelium and vascular smooth muscle cells,[31] with many of the hemodynamic changes observed in normal pregnancy being mimicked in nonpregnant animals chronically exposed to estrogen.[32] Both the endothelium and vascular smooth muscle cells express receptors for estrogen and progesterone[33] through which they can regulate vascular tone.[34] Therefore, they are likely to influence arterial stiffness through effects on mean arterial pressure, as well as structural changes to elastin, collagen, and smooth muscle in the arterial wall.[3,35] Progesterone has often be thought to have opposing vascular effects to estradiol, although it has favorable vascular effects in vitro[3] and in vivo.[34] However, despite the increased serum estradiol and progesterone concentrations with advanced gestation, we report an increase in arterial stiffness in the third trimester that we postulate is attributed to factors other than sex steroids.

Consistent with previous cross-sectional studies, all of the variables of systemic and central arterial stiffness measured were higher in women with preeclampsia.[15,16,36] We cannot exclude the possibility that medication influenced the collected data, because the effect of antihypertensive agents on PWV and augmentation index has not specifically been studied during pregnancy. Given that calcium channel blockers[37] and β-blockers[38] reduce PWV in nonpregnant populations, it seems likely that the increase in arterial stiffness observed in women with preeclampsia would have been even greater if these women were not taking antihypertensive agents.

The present study contrasts with a cross-sectional study that reported no difference in arterial stiffness assessed by augmentation index in women with a history of preeclampsia.[39] However this study was performed on average 5 to 6 years after the index pregnancy, and it is possible that applanation tonometry is not sensitive enough to detect more subtle remote effects. Similarly, Spasojevic et al[15] found no difference in the augmentation index between women with preeclampsia and healthy pregnant women at a 6-week postpartum visit. In our study, we performed a comprehensive assessment of arterial function and demonstrated that augmentation index and carotid-femoral PWV remained elevated at 7 weeks in women with preeclampsia compared with women with uncomplicated pregnancies.

Interestingly, carotid-radial PWV, unlike our other measures of arterial stiffness, had normalized by 7 weeks postpartum. Carotid-radial PWV is partly determined by the muscular brachial artery, whereas carotid-femoral PWV is determined by the more elastic aorta. Carotid-radial PWV is, therefore, susceptible to changes in both vascular smooth muscle tone and smooth muscle remodeling. It is, therefore, plausible that the increase in carotid-radial PWV in preeclampsia and pregnancy is in part attributed to an effect on smooth muscle function that may normalize more rapidly postpartum than any effect on the extracellular elastin-collagen matrix of the aorta. Other conditions, eg, diabetes mellitus and ageing, are known to have preferential effects on central rather than peripheral arteries,[40] and it is, therefore, perhaps not surprising that vascular remodeling in pregnancy similarly does not occur in a uniform manner.

Carotid-femoral PWV is recognized as the gold standard measure of arterial stiffness, as stated in the recent expert consensus document on the measurement of arterial stiffness.[1] In our cohort, carotid-femoral PWV remained elevated at 7 weeks postpartum, suggesting that the effects of preeclampsia on vascular structure and function extend beyond pregnancy. If arterial stiffness remains elevated in later life, this may in part contribute to the increased risk of cardiovascular and cerebrovascular diseases.[41]

Abnormalities of arterial structure and function were associated with higher postpartum blood pressures, although these women were no longer hypertensive, with blood pressures within the normal range. It is not possible from our studies to determine whether raised blood pressure during preeclampsia is a cause or a consequence of increased arterial stiffness. There is now good evidence to suggest that aortic stiffness is an independent predictor of progression to hypertension even in young nonhypertensive individuals,[42] with endothelial function being inversely related to arterial stiffness in healthy volunteers.[43] We, therefore, believe that in preeclampsia endothelial dysfunction increases aortic stiffness, which, in turn, causes an increase in blood pressure.

Alternative interpretations of our findings are possible, and, in particular, we cannot discount that changes in arterial stiffness occur as a consequence of prolonged hypertension in preeclampsia, nor can we be certain that changes in arterial stiffness at 7 weeks postpartum persist long term. A prospective study in which blood pressure and arterial stiffness were determined in a very large cohort of pregnant woman before the onset of preeclampsia with long-term postpartum follow-up would be required to address these issues.

Perspectives

In this longitudinal study, we have demonstrated that the augmentation index decreases during the luteal phase of the menstrual cycle with arterial stiffness rising from the midtrimester of pregnancy to term. The factors regulating these changes in arterial stiffness have yet to be identified. However, preeclampsia is associated with increased arterial stiffness, and this persists into the postpartum period. Increased arterial stiffness, therefore, appears to be a feature of preeclampsia that extends beyond pregnancy and suggests an abnormality of vascular structure and function associated with this condition, perhaps contributing to its adverse cardiovascular outcomes.

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Disclosures

None.

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human reproduction ORIGINAL ARTICLE *Gynaecology*

Influence of menstrual cycle on circulating endothelial progenitor cells

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BACKGROUND: Endothelial progenitor cells (EPCs) are circulating mononuclear cells that participate in angiogenesis. The aim of this study was to determine the influence of the menstrual cycle on the number and function of EPCs, and to investigate their relationship with circulating concentrations of sex steroids and inflammatory mediators.

METHODS: Ten healthy nulliparous, premenopausal, non-smoking women with regular menses were studied over a single menstrual cycle. Venepuncture was performed in the menstrual, follicular, peri-ovulatory and luteal phases. EPCs were quantified by flow cytometry ($CD133^+CD34^+KDR^+$ phenotype) and the colony-forming unit (CFU-EPC) functional assay. Circulating concentrations of estradiol, progesterone and inflammatory mediators (TNF-α, IL-6, sICAM-1 and VEGF) were measured by immunoassays.

RESULTS: The numbers of $CD133^+CD34^+KDR^+$ cells were higher in the follicular phase ($0.99 \pm 0.3 \times 10^6$ cells/l) compared with the peri-ovulatory phase ($0.29 \pm 0.1 \times 10^6$ cells/l; $P < 0.05$). In contrast, the numbers of CFU-EPCs did not vary over the menstrual cycle. There were no correlations between EPCs and concentrations of either circulating sex steroids or inflammatory mediators.

CONCLUSIONS: $CD133^+CD34^+KDR^+$ cells but not CFU-EPCs vary during the menstrual cycle. Our findings suggest a potential role for circulating EPCs in the normal cycle of physiological angiogenesis and repair of the uterine endometrium that is independent of circulating sex steroids or inflammatory mediators.

Key words: enothelial progenitor cells / menstrual cycle / angiogenesis / endometrium

Introduction

During the normal menstrual cycle, the uterine endometrium undergoes a unique cycle of physiological angiogenesis and endothelial repair. These processes begin in the proliferative phase and continue into the secretory phase of the cycle and are under tight hormonal and inflammatory control (Jabbour *et al.*, 2006). Until recently, endometrial angiogenesis was thought to occur primarily from new vessels sprouting via recruitment of local endothelial cells from neighbouring blood vessels (Risau, 1995; Gambino *et al.*, 2002). However recently, an alternative or complementary mechanism involving circulating endothelial progenitor cells (EPCs) has been proposed in the control and regulation of endometrial angiogenesis (Mints *et al.*, 2008).

EPCs are circulating mononuclear cells derived from the bone marrow, which have the potential to proliferate, migrate and differentiate into mature endothelial cells. Increasingly they are recognized as having an important role in mediating vascular endothelial repair and angiogenesis (Urbich and Dimmeler, 2004; Robb *et al.*, 2007). A role for EPCs in physiological endometrial angiogenesis was first implicated by Asahara *et al.* (1999) who demonstrated localization of EPCs within endometrial vasculature and stroma after induced ovulation in mice. More recently, an average 6% of endometrial endothelial cells were found to be donor derived after a haematological stem cell transplant in a mouse model (Bratincsak *et al.*, 2007). These animal studies are supported by clinical studies in women demonstrating that stromal and endothelial endometrial cells can originate from donor-derived bone marrow cells (Risau, 1995; Taylor, 2004).

Currently there is a lack of consensus on the definitive EPC phenotype and a variety of surface markers and functional assays are being

used to assess this progenitor population. This makes comparison between different clinical studies difficult. Although groups are increasingly quantifying EPCs by surface expression of phenotypic markers ($CD133^+CD34^+KDR^+$), the colony-forming unit endothelial progenitor cell assay (CFU-EPC) represents a functional assessment that quantifies the ability of putative endothelial progenitors to form colonies (Hill *et al.*, 2003). To date, relatively few clinical studies have either used both approaches or compared EPC phenotype and function.

Local factors including estradiol, tumour necrosis factor-α (TNF-α), interleukin (IL)-6, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule (ICAM)-1 are implicated in the regulation of endometrial angiogenesis (Kapiteijn *et al.*, 2001; Jabbour *et al.*, 2006; Jasper *et al.*, 2007). Many of these factors also appear to be involved in EPC mobilization and trafficking in mice models (Rafii *et al.*, 2002; Iwakura *et al.*, 2003; Hamada *et al.*, 2006; Iwakura *et al.*, 2006) and human studies (Naiyer *et al.*, 1999; Kalka *et al.*, 2000; Bulut *et al.*, 2007; Foresta *et al.*, 2007; Grisar *et al.*, 2007; Fan *et al.*, 2008). However, whether cyclical systemic variation of these factors during the menstrual cycle is associated with changes in phenotypic or functional circulating EPCs is unknown. The aims of this study were therefore to determine the influence of the menstrual cycle on the number and function of circulating EPCs, and to investigate how their number and function vary with cyclical variation of circulating sex steroids or inflammatory mediators.

Materials and Methods

Subjects

Ten healthy, non-smoking, nulliparous women with at least a 2 month history of normal regular menstrual cycles gave written informed consent and were recruited to the study. Exclusion criteria included use of hormonal contraception, infection, hypertension or other significant past medical history, regular medication, or a history of heavy menstrual bleeding or dysmennorhoea. All the study group had confirmed ovulation, defined as Day (D) 21 serum progesterone >30 nmol/l. The study was approved by the local research ethics committee and undertaken in accordance with the Declaration of Helsinki.

Visit schedule

Volunteers abstained from alcohol, caffeine and tobacco for 12 h and fasted for 4 h prior to attendance. Participants attended for four visits during a single menstrual cycle [D 1–3 (menstrual), D 6–8 (mid-follicular), D 13–15 (peri-ovulatory) and D 20–22 (mid-luteal)]. At the first visit, blood pressure and heart rate were recorded in duplicate using an automated sphygmomanometer (Micro life 3BTO-A) following a 15 min supine rest. Height (m) and weight (kg) were also measured to allow calculation of body mass index (BMI; kg/m^2). At each visit, 50 ml of peripheral venous blood was collected from the antecubital fossa: 30 ml was anti-coagulated with EDTA (Sarstedt-Monovette, Germany) for full blood count, flow cytometric analysis and CFU-EPCs culture; 10 ml was anti-coagulated with EDTA and centrifuged at 2000g with plasma being stored at −80°C until subsequent analysis; and the remaining 10 ml was collected in a serum tube for hormonal analysis.

Flow cytometry

EPCs were quantified by phenotype in whole blood samples using flow cytometry. Briefly, cells were directly stained and analysed for phenotypic expression of surface antigens using anti-human monoclonal antibodies conjugated to phycoerythrin (PE), fluorescein isothiocynate (FITC) or allophycocyanin (APC). Whole blood (200 μl) was stained with antibodies [anti-CD34-FITC (Becton Dickinson, Oxford, UK), anti-CD133-PE (Miltenyl Biotech, Germany) and anti-KDR(VEGFR2)-APC (R&D Systems, Minneapolis, USA)] for 20 min in the dark at room temperature. Appropriate negative unstained controls and isotype-matched anti-IgG_1-APC (R&D Systems) were used to establish positive stain boundaries. Erythrocytes were lysed (UTI-lyse™, Dako). Next, samples were centrifuged at 1500*g* for 5 min at room temperature, and cells were washed and re-suspended in 500 μl of phosphate-buffered saline (PBS) without cations (Sigma-Aldrich, Poole, UK). For FACS analysis, 80 000 events were acquired in the lymphocyte region (as determined by characteristic forward and side scatter profile) using a FACSCalibur® analyser (Becton Dickinson). EPCs were quantified based on the percentage of $CD34^+$ $CD133^+KDR^+$ triple positive leucocytes and expressed as number of cells per litre of blood.

CFU-EPC assay

Based on the assay described by Hill *et al.* (2003), functional EPCs were quantified using the CFU-EPC assay and commercial kit reagents according to the manufacturer's recommendations (StemCell Technologies, UK). Briefly, blood samples were diluted with PBS without cations (Sigma-Aldrich) and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Paque (Histopaque, Sigma-Aldrich). PBMCs were washed twice with PBS and once with culture medium and re-suspended at 5×10^6 cells/ml in Complete Culture Medium (EndoCult Supplement mixed with EndoCult Basal Medium and PenStrep, containing penicillin and streptomycin, Stem Cell Technologies, UK). Cells were then plated at 2 ml/well in a 6-well fibronectin-coated plate (Becton Dickinson) and incubated at 37°C, 5% CO_2 with 95% humidity for 2 days. The non-adherent cells were then harvested and transferred to fibronectin-coated 24-well plate at 1×10^6 cells/well (Becton Dickinson) for a further 3 days. Colonies (CFU-EPC, early outgrowth colony-forming endothelial progenitor cells; Stem Cell Technologies) were defined using the published method as a central core of 'round' cells surrounded by elongated 'sprouting' cells at the periphery. Colonies were counted in a minimum of two wells per sample by observers unaware of the subjects' clinical profiles and the results were expressed as mean numbers of CFU-EPCs per 1×10^6 cells plated.

Uptake of acetylated LDL and immunophenotyping

To confirm endothelial-cell lineage, colonies were stained directly using acetylated LDL and co-stained with Lectin (*Ulex europaeus I agglutinin*) as previously described (Kalka *et al.*, 2000). Briefly, cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylcarbocyanine-labelled acetylated LDL (Dil-AcLDL (Serotech); 1:100 in Endocult) for 4 h, washed in PBS and fixed with ice-cold methanol for 10 min. Colonies were stained for 1 h with FITC-labelled lectin from *Ulex europaeus* (Sigma; 1:100 in PBS), counterstained with DAPI (Sigma) and mounted with Permafluor (Immunotech).

For CD105 (endoglin) and CD146 staining, fixed colonies were permeabilized and blocked in a solution of 10% goat serum (Autogen Bioclear), 1% BSA (Sigma) and 0.02% IGEPAL/NP40 (Sigma). Next, colonies were incubated with primary murine monoclonal antibodies against human CD105 (1:500, BD Transduction Laboratories) or CD146 (1:200, Springbioscience) for 2 h at room temperature. A secondary polyclonal goat anti-mouse biotinylated antibody (DAKO) was added for 30 min followed by streptavidin-Alexa Fluor 546 or streptavidin-Alexa Fluor 488 (Molecular probes) for 1 h in the dark. Nuclei were counterstained and colonies were mounted as above. Between each step the

slides were washed with PBS for 5 min. Human umbilical vein endothelial cells (HUVECs) were used as positive controls for all of the endothelial markers. Staining was performed without the primary antibody as a negative control for each antibody. In addition, a human colon cell-line (HT29) population was used as a negative control for the endothelial markers. Phase-contrast images of the same CFU-EPCs were used to confirm colony morphology. All images are representative of at least three different subjects. A Zeiss 'LSM 510 Meta' confocal microscope, with ×40 oil immersion objective was used to visualize the stained cells. Image capture was performed with LSM 510 meta-analysis computer software.

Hormone and inflammatory marker assays

Serum concentrations of estradiol, progesterone, lutenizing hormone (LH) and follicle-stimulating hormone (FSH) were measured using the Siemens Medical Solutions Centaur and Abbott Architect immunoassay systems. Commercial enzyme-linked immunosorbent assays (ELISAs) were used to measure plasma concentrations of TNF-α (measures total TNF-α comprising free TNF-α and TNF-α bound to its soluble receptors), IL-6, sICAM-1 (all R&D Systems, detection limits 0.1 pg/ml, 0.039 pg/ml and 0.35 ng/ml, respectively) and VEGF (Calbiochem, Darmstadt, Germany, detection limit 9.0 pg/ml).

Statistical analysis

Continuous variables are reported as mean ± SEM. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni's post-tests for multiple comparisons. Non-parametric Friedman analyses were used where appropriate. Correlation coefficients were calculated using Pearson or Spearman analyses for parametric and non-parametric data, respectively. All calculations were performed using GraphPad Prism (GraphPad Software, USA). Statistical significance was taken at 5%.

Results

All women (age, 31.4 ± 2.0 year) were of normal body composition (BMI, 23.4 ± 0.7 kg/m^2) and had regular menstrual cycles (cycle length 28 ± 0.4 days). Baseline systolic and diastolic blood pressures and heart rate, measured at D 1–3 of the cycle, were 110 ± 2 mmHg, 67 ± 2 mmHg and 60 ± 2 bpm, respectively.

Phenotypic and functional EPCs during the menstrual cycle

Triple positive ($CD34^+CD133^+KDR^+$) EPCs varied during the menstrual cycle ($P = 0.04$) with mid-follicular levels ($0.99 \pm 0.3 \times 10^6$ cells/l) being 3-fold higher than peri-ovulatory levels ($0.29 \pm 0.1 \times 10^6$ cells/l) (Fig. 1A; $P < 0.05$). A similar trend was seen for $CD34^+KDR^+$ cells (data not shown).

In contrast, there was no variation in numbers of CFU-EPCs during the menstrual cycle (Fig. 1B; $P > 0.05$). Direct staining confirmed that CFU-EPCs, like mature endothelial cells, bind lectin, integrate acetylated LDL and are positive for the endothelial markers CD105 and CD146 (Figs 2 and 3, respectively). There was no correlation between EPCs quantified by phenotype ($CD34^+CD133^+KDR^+$) and number of functional CFU-EPCs during the menstrual cycle.

Sex steroids and inflammatory mediators

There was normal cyclical variation in circulating pituitary and ovarian hormones with peri-ovulatory peaks in serum LH and FSH concentrations, and peri-ovulatory and mid-luteal peaks in serum estradiol and progesterone concentrations, respectively (Table I). Concentrations of plasma TNF-α ($P = 0.05$) but not IL-6, sICAM-1 or VEGF ($P = 0.51$, $P = 0.36$, $P = 0.84$, respectively) varied over the menstrual cycle. There was no relationship between functional or phenotypic EPCs and circulating levels of estradiol, progesterone, sICAM-1 (all $r^2 < 0.06$), IL-6 ($r = 0.06$) or VEGF ($r = 0.01$) at any phase of the menstrual cycle. A weak correlation was seen between plasma levels of TNF-α and concentration of phenotypic EPCs ($r = 0.33$, $P = 0.04$).

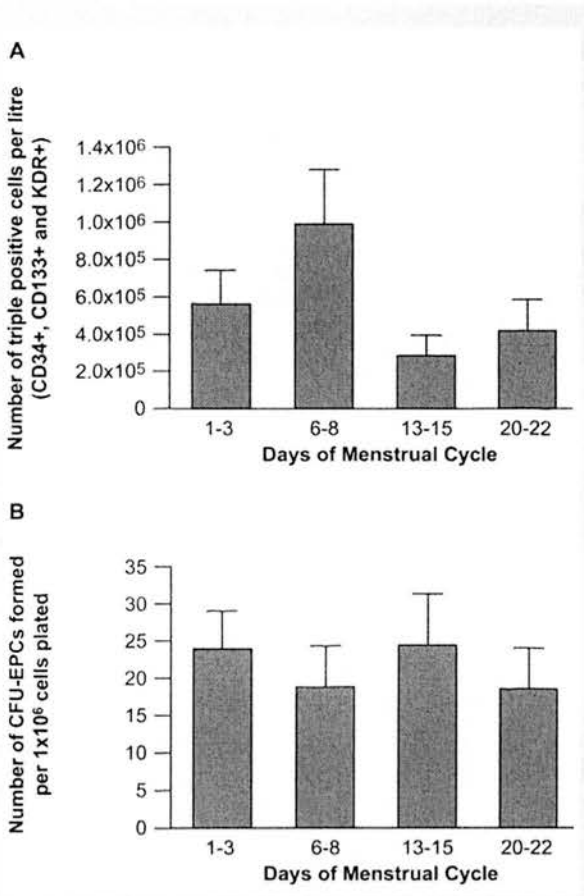

Figure 1 (**A**) Concentrations of circulating triple positive $CD133^{+-}$ $CD34^+KDR^+$ EPCs varied throughout the menstrual cycle ($P = 0.04$, one-way ANOVA with repeated measures) with mid-follicular levels ($0.99 \pm 0.3 \times 10^6$ cells/l) being 3-fold higher than peri-ovulatory levels ($0.29 \pm 0.1 \times 10^6$ cells/l, $P < 0.05$, Bonferroni's post-test for multiple comparisons).
(**B**) Numbers of CFU-EPCs do not vary throughout the menstrual cycle (one-way ANOVA with repeated measures) ($P > 0.05$).

Discussion

This study demonstrates that numbers of circulating EPCs quantified by phenotype ($CD34^+CD133^+KDR^+$ cells) vary during the menstrual cycle, peaking in the mid-follicular phase. In contrast, EPCs quantified

by the functional CFU-EPC assay did not vary during the menstrual cycle. During the normal menstrual cycle, we propose that mobilized $CD34^+CD133^+KDR^+$ cells have the potential to contribute to endometrial angiogenesis during the mid-follicular phase of the cycle (Girling and Rogers, 2005). Given that there was no correlation between EPCs quantified by phenotype or function and the levels of circulating sex hormones or inflammatory mediators measured, this raises the possibility of an endometrial specific factor being present in mid-follicular phase endometrium, which has either the potential to mobilize EPCs or to remove them from the peripheral circulation into endometrium.

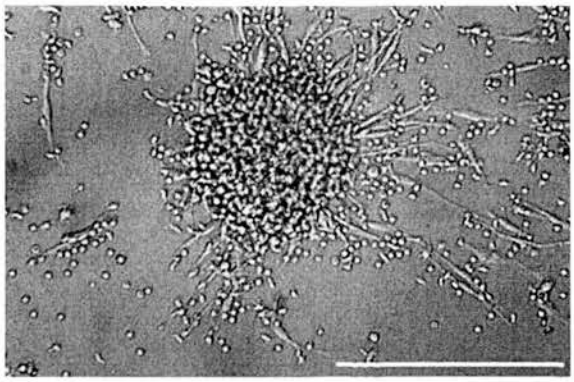

Figure 2 Typical colony-forming unit.
A typical colony-forming unit (CFU-EPC) with a characteristic core of round cells and sprouting spindle cells at the periphery (×100 magnification).

The present study is the first to investigate the influence of the menstrual cycle on both phenotypic and functional EPCs. Our finding that $CD34^+CD133^+KDR^+$ EPCs peak during the mid-follicular phase differs from two recent studies of EPCs during the menstrual cycle (Matsubara *et al.*, 2006; Fadini *et al.*, 2008). A variety of factors, in particular the differing methodologies employed and timing of sampling, may account for these seemingly discrepant findings. Fadini *et al.* (2008) demonstrated a similar 2-fold increase in $CD34^+KDR^+$ numbers during the menstrual cycle, but the peak seemed to occur in the peri-ovulatory phase rather than earlier during the follicular phase as in our study. However, Fadini *et al.* (2008) did not specify the day of sampling so it is possible that this peak occurred between Day 6 and 12 of the menstrual cycle which would therefore be entirely consistent with our findings. In the cross-sectional study by Matsubara *et al.* (2006), they found that EPCs were increased in the luteal phase compared with the follicular phase of the cycle in women. They quantified the proliferative capacity of EPCs using adherent peripheral blood mononuclear cells that tested positive for Ac-LDL and lectin after 7 days in culture, and did not use the EPC-CFU assay that we have described here. Moreover, although Matsubara *et al.* (2006) compared EPCs during the follicular and luteal phase of the cycle, it is again unclear on which day the samples were taken in their cross-sectional study. From our longitudinal study, in which samples were taken at four consecutive time-points within a single cycle, there are clear differences in concentrations of $CD34^+CD133^+KDR^+$ EPCs depending on the day of sampling. This variation will be magnified if a broader time window of sampling

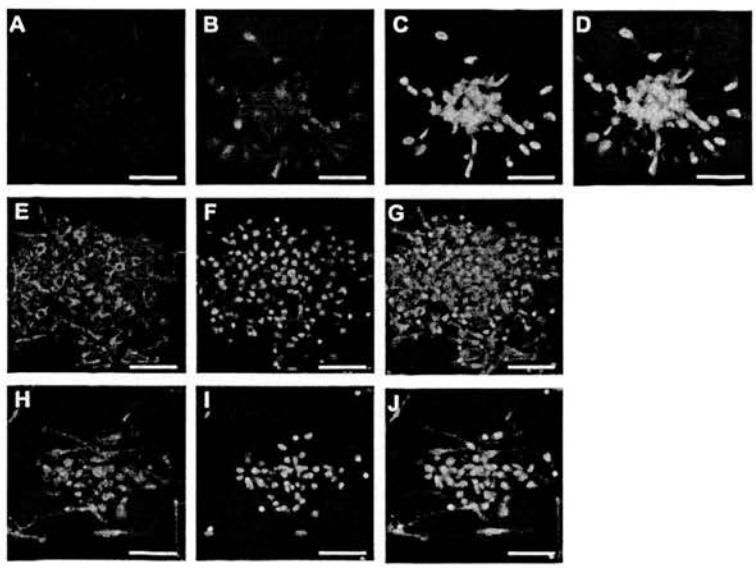

Figure 3 Characterization of CFU-EPCs.
Live CFU-EPCs exposed to DiI-AcLDL (**A**), stained with FITC-lectin (*Ulex europeaus*) (**B**), nuclear counterstain DAPI (**C**) and merged image (**D**). Fixed CFU-EPCs immunostained for the endothelial cell marker CD105 (**E**), nuclear counterstain (**F**) and merged image (**G**) and for CD146 (**H**), nuclear counterstain (**I**) and merged image (**J**) (×40 magnification).

Table I Hormonal and inflammatory profile of participants

	Day of menstrual cycle				Significance
	1–3	6–8	13–15	20–22	
Day at sampling	2 ± 0.2	7 ± 0.2	14 ± 0.3	21 ± 0.2	
LH (U/l)	5 ± 0.4	7 ± 0.4	32 ± 7.2	5 ± 0.5	$P < 0.0001$
FSH (U/l)	6 ± 0.4	6 ± 0.4	9 ± 1.9	3 ± 0.2	$P = 0.002$
Estradiol (pmol/l)	180 ± 21.1	318 ± 68.4	1342 ± 274.4	827 ± 93.4	$P < 0.0001$
Progesterone (nmol/l)	4 ± 0.3	3 ± 0.2	7 ± 2.1	47 ± 4.5	$P < 0.0001$
sICAM-1 (ng/ml)	214 ± 15.2	193 ± 11.6	203 ± 8.1	199 ± 11.8	$P = 0.36$
VEGF (pg/ml)	42 ± 16.0	44 ± 14.8	39 ± 11.3	93 ± 60.4	$P = 0.84$
TNF-α (pg/ml)[a]	0.73 (0–3)	0.41 (0–0.9)	0.075 (0–1)	0.0 (0–0.4)	$P = 0.05$
IL-6 (pg/ml)[a]	0.13 (0.1–0.2)	0.11 (0.1–0.2)	0.095 (0.06–1)	0.14 (0.07–0.5)	$P = 0.51$

Circulating LH, FSH, estradiol and progesterone varied significantly throughout the menstrual cycle. Plasma TNF α concentrations decreased, while levels of IL-6, sICAM-1 and VEGF did not vary significantly. Values reported as mean ± SEM or [a]median with inter-quartile ranges.

was used, and by the use of multiple subjects because of the potential for inter-subject variability.

The exact phenotype of EPCs has been debated since the original publication by Asahara *et al.* (1999) in which it was demonstrated that $CD34^+$ cells extracted from circulating peripheral blood formed new blood vessels in culture and contributed to angiogenesis in a mouse model of ischaemia. The purity of this cell population was only 15% raising the possibility that a variety of other circulating cell types may also have angiogenic potential. The current consensus is that mononuclear cells co-expressing CD34, CD133 and KDR (so-called 'triple positive' cells) constitute the most likely phenotype of circulating endothelial progenitors. However, these cells are rare in normal peripheral blood, constituting between 0.01 and 0.0001% of the circulating leukocyte population, making their accurate quantification by flow cytometry difficult. As a result, assays based on the quantification of functional populations of EPCs with colony-forming activity in cell culture (CFU-EPC) have emerged as alternatives (Hill *et al.*, 2003).

The validity of the CFU-EPC assay in quantifying EPCs has recently been challenged by studies showing that the CFU-EPC cells are derived from monocyte-like cells and not the circulating $CD34^+$ haemangioblast (Prater *et al.*, 2007; Rohde *et al.*, 2007; Yoder *et al.*, 2007). Our finding of a lack of correlation between concentrations of $CD34^+CD133^+KDR^+$ EPCs and EPCs quantified by the CFU assay is therefore important. Currently, these two methods are used interchangeably in clinical studies and are in general thought to be measuring the same endothelial progenitor, derived from $CD34^+$ pluripotent cells, using different techniques. This is clearly not the case as our study demonstrates. Thus, cells quantified by flow cytometry (phenotype) and cell culture (CFU-EPC) are likely to be different cell populations with differing biological functions and progenitor potential (Tura *et al.*, 2007). Ingram *et al.* (2004) recently have described a new EPC population in human umbilical cord blood called the 'late outgrowth endothelial progenitor cell' with a high proliferative capacity and the ability to express endothelial markers and form tubules *in vitro*. This population arises from $CD34^+$ cells. Thus, the $CD34^+CD133^+KDR^+$ EPCs may correlate with this late-outgrowth assay.

We demonstrate that $CD34^+CD133^+KDR^+$ EPCs peak during the mid-follicular phase of the menstrual cycle. In our study, this effect does not appear to be driven by the circulating sex hormones or inflammatory factors studied. The lack of correlation of EPCs with serum estradiol concentrations differs from a study by Sugawara *et al.* in which they found that during pregnancy, there was a clear correlation between estradiol and EPCs quantified by a modified CFU-EPC assay (Sugawara *et al.*, 2005). Differences in methodology or the higher serum concentrations of estradiol in pregnancy compared with the menstrual cycle could account for these differences. In our study, there was no correlation between concentrations of the circulating inflammatory mediators studied and EPCs. A number of factors may explain this. First, unlike $CD34^+CD133^+KDR^+$ EPCs, there was no cyclical variation in concentration of the inflammatory mediators IL-6, sICAM-1 and VEGF studied over the menstrual cycle. Although, there was a decrease in soluble TNF-α concentration over the cycle and a weak correlation with numbers of $CD34^+CD133^+KDR^+$ EPCs, the absolute levels of TNF-α were almost 10× lower than in conditions such as rheumatoid arthritis and cardiac failure, where strong correlations with EPCs have been demonstrated (Valgimigli *et al.*, 2004; Grisar *et al.*, 2005). It is therefore not surprising that concentrations of the circulating inflammatory mediators and EPCs were not correlated. Moreover, the small numbers in our study may also be a contributing factor. Second, mobilization of EPCs may be controlled by local rather than systemic levels of inflammatory mediators. Mobilization of EPCs in response to increased local but not systemic concentrations of VEGF and stromal cell-derived growth factor has recently been demonstrated in animal and human limb ischaemia models (Hur *et al.*, 2007; Oh *et al.*, 2007). We measured systemic and not local levels of VEGF. This may account for the lack of correlation between VEGF and EPCs demonstrated in this study. Finally, other factors thought to be involved in regulation of endometrial angiogenesis, including the angiopoetins (Girling and Rogers, 2005; Saito *et al.*, 2007), may play a role in EPC mobilization and recruitment (Mints *et al.*, 2007) during the mid-follicular phase of the menstrual cycle.

In summary, we have demonstrated that during the normal menstrual cycle, there is cyclical variation of phenotypic triple positive

EPCs. Their peak in the mid-follicular phase suggests a potential role for circulating EPCs in the normal cycle of physiological angiogenesis and repair of the uterine endometrium. It would be of interest to determine whether pathologies such as heavy menstrual bleeding and endometriosis are associated with dysregulation of EPC number or function.

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